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FINAL REPORT

No. 1504 F

on

DEVELOPMENT OF BACTERIAL SENSING INSTRUMENTATION FOR SPACECRAFT WATER SYSTEMS

to

NASA/MANNED SPACECRAFT CENTER

R&D Procurement Branch

Houston, Texas 77058

Contract NAS 9-10432

[illegible] November 1970

AEROJET MEDICAL AND BIOLOGICAL SYSTEMS
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Section 1

SUMMARY

The porphyrin initiated chemiluminescence technique forms the basis of an instrument which has been developed for monitoring water sterility in spacecraft water storage and supply systems. The instrumentation which has been developed permits differentiation between living and dead organisms by comparing the chemiluminescent signals of incubated and unincubated water samples; a higher signal for the incubated sample is used to indicate non-sterility of the water supply. The system selected for the breadboard prototype is both sensitive and rapid and has the potential of being developed into a compact lightweight instrument capable of operation in a zero gravity environment.

Using E. coli and S. marcescens as test organisms, demonstrated sensitivities of 20 to 30 viable cells/ml and 75 to 150 total cells/ml (100 ml sample) have been achieved with this instrument. Evidence is presented that further increases in sensitivity may be achieved by an improved reactor design which permits more optimum coupling to the photomultiplier tube; sensitivity may also be increased by increasing sample size.

The protocol which was found to yield the maximum sensitivity for total (viable + non-viable) and viable cells entailed processing the water sample through two cycles of concentration, washing and backwashing with an intermediate incubation step in the viable cycle. Dextrose broth served as the wash and backwashing fluid in the first cycle and 2M urea in the second. The sterility of the system was maintained by flushing with 8M urea in between runs. The processing time for monitoring total cell count in a 100 ml water sample was 51 minutes, and for differentiation of viable from non-viable cells, an additional 2-hour incubation period was required.

In a study of the effect of water immersion on sensitivity for total cells, a slight decrease (i.e., 25% or less) was observed for organisms stored at either refrigeration ($\sim 5^{\circ}C$ for one week) or ambient (24 hours) temperature.

Reagent shelf life studies indicated that 4 of the required 5 reagents (luminol, H_2O_2, 2M and 8M urea) for this system exhibited no loss in efficacy on storage for 5 weeks at ambient temperature in the light. Dextrose broth exhibited some evidence of deterioration under these conditions of storage.

An analysis of eleven regenerated water samples received from the Space Simulator Station at McDonnell-Douglas indicated viable counts ranging from ~ 0 to $> 10^3$ cells/ml and total cell counts, in all cases, in excess of 10^3 cells/ml. The dominant organism isolated from these water samples was assigned to the Achromobacter group on the basis of microbiological and biochemical tests.

The soluble signals (i.e., signal obtained on reacting filtrate with luminol-hydrogen peroxide reagent) for each of the samples tested were found to be 4- to 100-fold greater than that exhibited by fresh glass distilled water. Evidence is presented which indicates this soluble fraction is due principally to porphyrins from lysed organisms.

Section 2

INTRODUCTION

This is the final report of progress in "Development of Bacterial Sensing Instrumentation for Spacecraft Water Systems" and is submitted in accordance with the requirements of Contract No. NAS 9-10432.

The general objective of the present effort was to establish guidelines for a program to develop instrumentation for bacterial monitoring of spacecraft water systems. The instrumentation envisaged was to be capable of providing a continuous indication of water system sterility.

A more specific objective was to evaluate the feasibility of the porphyrin-initiated chemiluminescence technique for the intended application, using a suitable breadboard.

The principle of detection selected was based on measuring the increase in chemiluminescence produced by the catalytic action of bacterial porphyrins on a luminol*-hydrogen peroxide mixture. These metal porphyrins are ubiquitous in nature occurring in the electron transport system of most microorganisms. The reaction is virtually instantaneous and occurs immediately on mixing the bacterial suspension with the aqueous reagents. Mixing is carried out within view of a photomultiplier which monitors the light emitted by the reaction. The signals which are generated are directly proportional to the number of bacteria present. Since dead as well as live organisms can initiate luminescence, to differentiate between the two, chemiluminescent signals are obtained for both incubated and unincubated bacterial samples. A higher signal for the incubated sample indicates the presence of viable organisms and a cause for rejection of the water supply.

Several advantages of porphyrin over ATP** initiated chemiluminescence for bacterial monitoring is that the porphyrin approach

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*Luminol is 5-amino-2, 3-dihydro-1-4-phthalazinedione.

**ATP is adenosine triphosphate, a nucleotide present in living organisms; reaction of ATP with luciferin-luciferase reagent produces luminescence.

does not require prior extraction to initiate luminescence and it utilizes inexpensive off-the-shelf reagents which do not require refrigeration. These combined features permit utilization of a far simpler instrument design which is more readily adaptable to in-line, zero gravity operation.

The instrumentation developed under the present program and the results obtained are described in detail below.

Section 3

EXPERIMENTAL RESULTS

3.1 INSTRUMENTATION

A schematic representation of the breadboard instrumentation developed under the current program for sterility monitoring of spacecraft water is shown in Figure 1.

The unit shown can be used for monitoring either the total (viable + non-viable) cell count or for detecting the presence of viable organisms in a water sample. For determination of the latter, it is first necessary to obtain a baseline value on an unincubated sample processed in the same manner as the incubated sample except that the actual incubation has been omitted. The signal so obtained reflects the total cell population of viable and non-viable which may be present. If subsequent processing of a second sample through an incubation cycle produces a higher signal than the first, the presence of viable organisms is indicated.

The operational sequence for processing the unincubated sample is summarized in Table 1. It entails pumping the water sample through a filter (Filter No. 1, Figure 1) to concentrate the organisms. These organisms are then washed with Dextrose broth and then recovered by backwashing with the same medium. The backwash flow is maintained long enough to transport the organisms to the incubator which is held at 37°C. The organisms are then concentrated on a second filter (Filter No. 2, Figure 1) where they are washed free of nutrient with 2M urea*. The bacteria are backwashed off Filter No. 2 with 2M urea and react with luminol-H_2O_2 reagent in a glass reactor mounted in front of a photomultiplier tube. The emitted light is amplified and signal amplitude is recorded on a strip chart recorder.

The procedure used for the viable cycle is identical (see Table 2) except that a 2 hour incubation period is employed and a bactericide (5M urea) used as a flush between samples to maintain the sterility of the system.

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*The nutrient dextrose broth will produce a signal and so has to be removed prior to reaction of the bacteria with luminol-H_2O_2 reagent.

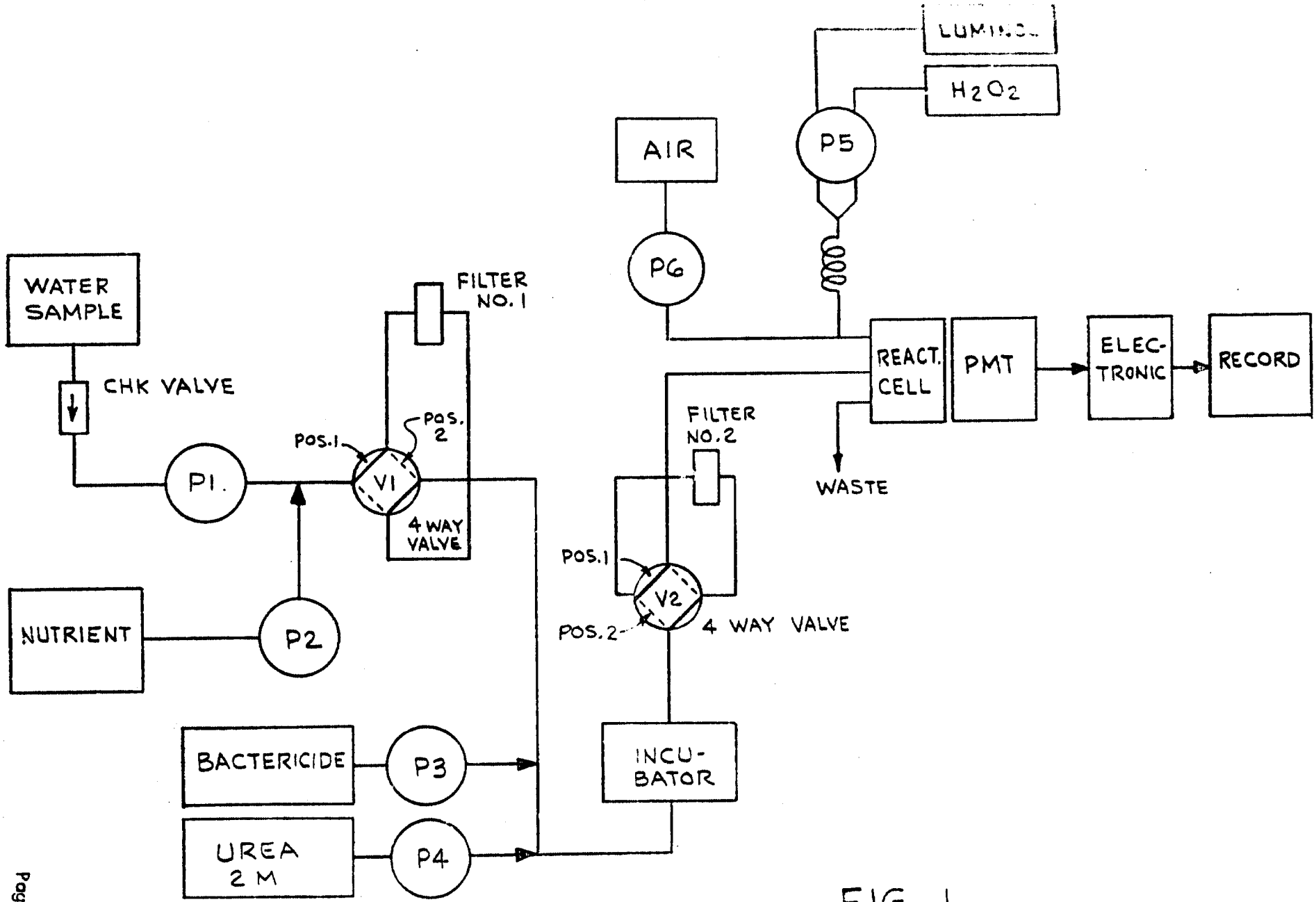

FIG. 1

SCHEMATIC OF WATER MONITOR

Table 1

OPERATIONAL SEQUENCE FOR OBTAINING TOTAL CELL COUNT
(Unincubated Sample, Stage 3)

Operation		Flow Rate	Flow Duration
1.	Concentration of Sample	4 ml/min.	25 min. *
2.	Wash (W_1) with Nutrient**	1 ml/min.	3 min.
3.	Backwash (BW_1) with Nutrient	1 ml/min.	3 min.
4.	Wash (W_2) with 2M Urea	1 ml/min.	10 min.
5.	Backwash (BW_2) with 2M Urea	1 ml/min.	5 min.
6.	Reagent Flow		
	Luminol	0.11 ml/min.	Continuous
	H_2O_2	0.24 ml/min.	Continuous
	Air	0.35 ml/min.	Continuous

*For 100 ml sample.

**Dextrose Broth.

Table 2

OPERATIONAL SEQUENCE FOR VIABLE CYCLE
(Incubated Sample, Stage 3 Protocol)

Operation		Flow Rate	Flow Duration
1.	Concentration of Sample	4 ml/min.	25 min. *
2.	Wash (W_1) with Nutrient**	1 ml/min.	3 min.
3.	Backwash (BW_1) with Nutrient	1 ml/min.	3 min.
4.	Incubate (2 hours)	-	-
5.	Wash (W_2) with 2M Urea	1 ml/min.	10 min.
6.	Backwash (BW_2) with 2M Urea	1 ml/min.	5 min.
7.	Bactericide	1 ml/min.	5 min.
8.	Reagent Flow		
	Luminol	0.11 ml/min.	Continuous
	H_2O_2	0.24 ml/min.	Continuous
	Air	0.35 ml/min.	Continuous

*For 100 ml sample.

**Dextrose Broth.

A detailed description of the operation of each of these cycles is contained in Appendix A.

3.1.1 SYSTEM LAYOUT

A photograph and sketch of the physical configuration of the breadboard unit are shown in Figures 2 and 3 respectively. All components are mounted to a single side of a base plate. This configuration permits rapid assembly as well as immediate access to all components for the purpose of adjustment or modification.

During the protocol development phase, maximum flexibility in sample manipulation was achieved by manual control of all valve and pump operations*. Having established the optimum timing sequence for sample processing during the incubated and unincubated cycles, changeover to program timers** for completely automatic operation is a simple matter and well within the state-of-the-art.

3.1.2 MAJOR COMPONENTS

A brief description of the major components contained in Figures 2 and 3 follow.

3.1.2.1 FLUID PUMPS

Independently operated Buchler Polystaltic pumps are used for all metering and fluid transport in the breadboard system. Desired flow rates are achieved by varying the rotor speed and/or varying the diameter of the Tygon tubes passing through the rollers. On-off control of liquid flow is accomplished by energizing and de-energizing the pump motor. The pump employs a spring-loaded pressure plate to maintain constant roller pressure against the pumping tubes and eliminates variations in delivery rates associated with tubing wear. When functioning properly, no check valves are required with this pump. The maintenance-free life of the pumping tubes is approximately 150-200 hours.

For flight-rated systems where weight is at a premium, the

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*Control panel toggle switches were used for manual initiation and termination of all valve and pump operations.

**Timing cams available from Precision Mechanics Corp., or Industrial Timer Corp., (N.J.).

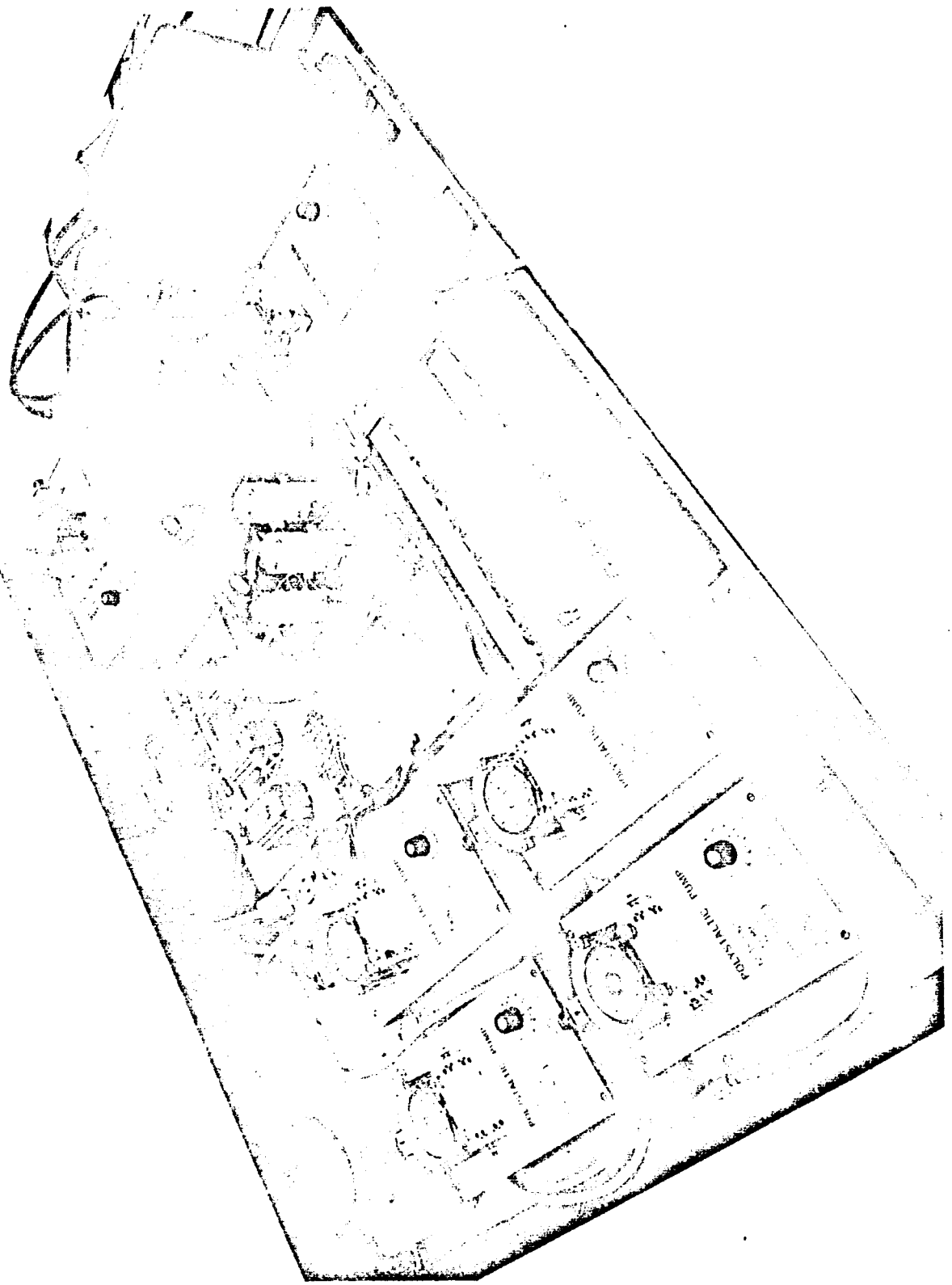


Figure 2. amb WATER MONITOR - Breadboard Model

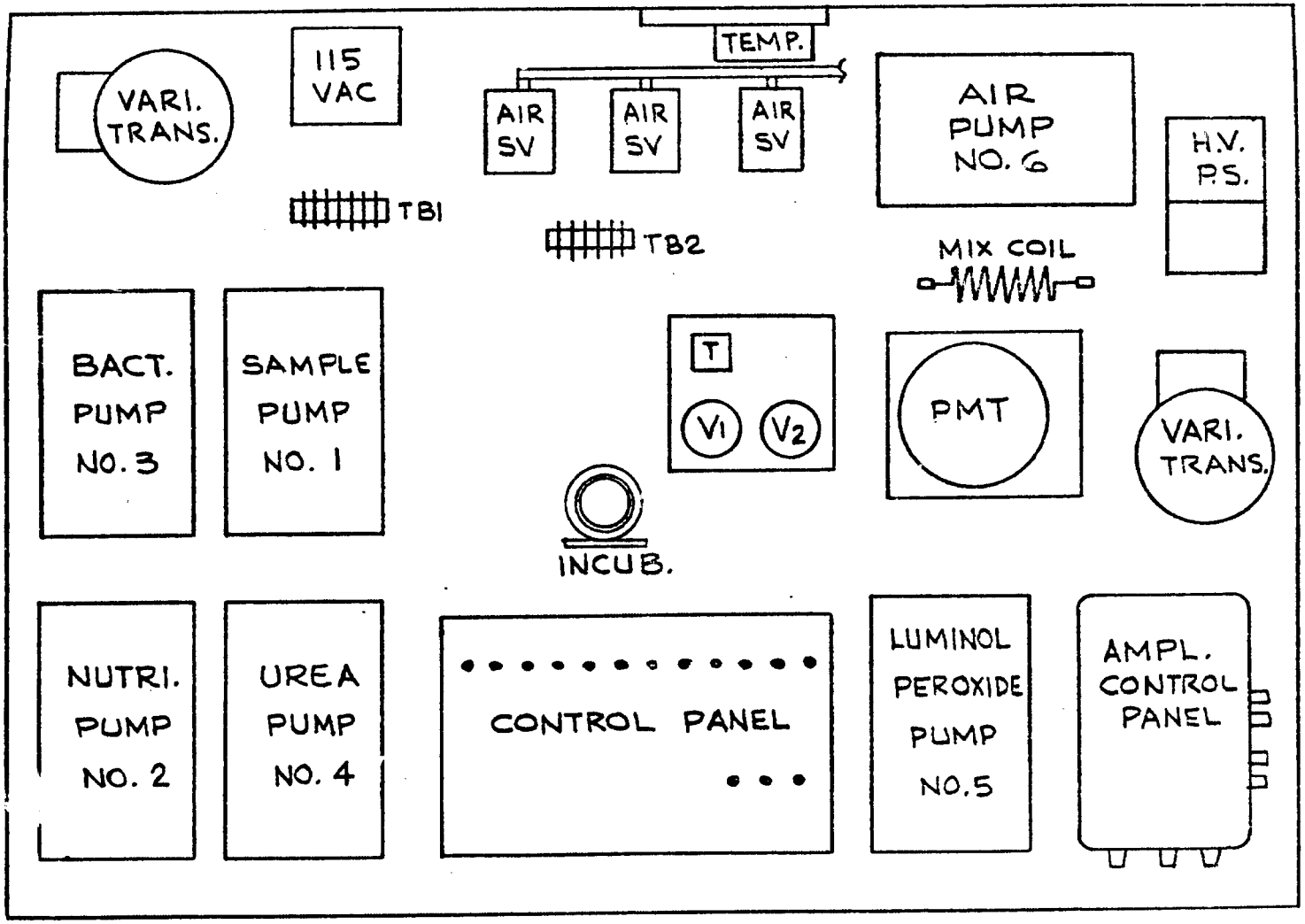


FIG. 3

SYSTEM LAYOUT

Buchler pumps could be replaced by small light-weight peristaltic pumps, such as those manufactured by Nebraska Medical Instruments, Inc., Omaha, Nebraska. Where maintenance-free operation on the order of several thousand hours or more are desired, use of a suitable positive displacement pump (stainless steel (316) and Teflon construction) would be recommended.

3.1.2.2 CONCENTRATING FILTERS

The filters used for concentrating the sample before and after incubation consist of the following:

	Type Filter Holder	Filter Material
Filter No. 1	25 mm dia. Micro-Syringe (Millipore) (4.0 cm^2 effective filtering area)	0.45 μ Acropor AN* (Gelman)
Filter No. 2	13 mm dia. Swinny (Millipore) (0.8 cm^2 effective filtering area)	0.45 μ Acropor AN (Gelman)

Filter No. 2, contains a KEL-F insert (Figure 4) to reduce the void-volume between this filter and the reactor. Since the sensitivity of the system depends on the bacterial concentration, maximum sensitivity is achieved if the liquid volume into which the bacteria are backwashed is at a minimum.

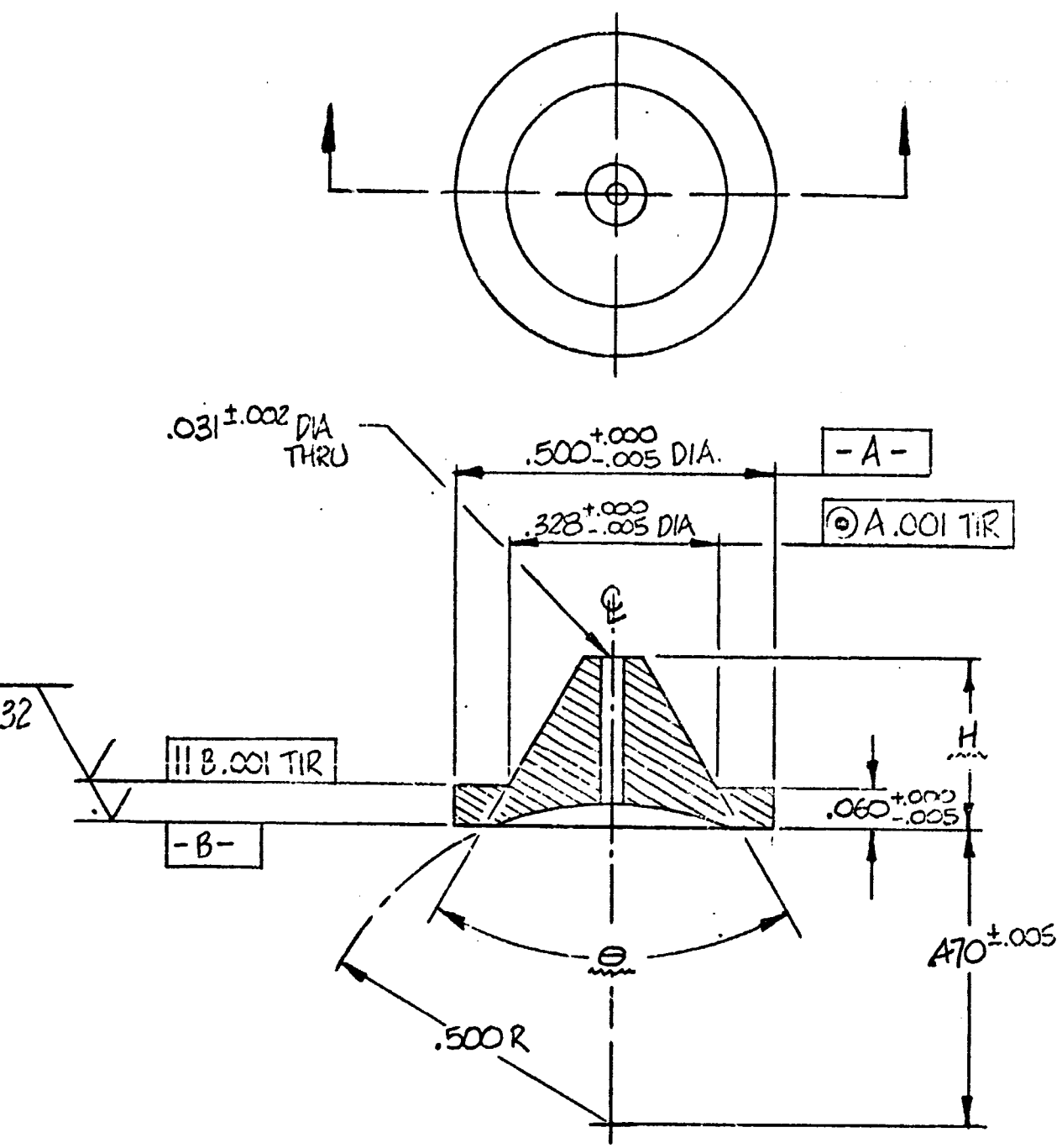
Bacterial recoveries from Acropor filter membranes were found to be superior to others tested. Presoaking of this filter in 8M urea for at least 15 minutes followed by rinsing in distilled water is required before use to remove objectionable finishing materials. Under normal operation filters could last for about a week before requiring replacement. The standard procedure employed during the present program was to replace these filters daily, however.

3.1.2.3 INCUBATOR

The results of laboratory studies indicated that aeration of the nutrient mixture during incubation was not required since this medium contained sufficient dissolved oxygen to support bacterial growth over the time interval in question. On the basis of these experiments, the incubator was simply a glass tube (4 ml volume) immersed in a thermostatically controlled environment (Figure 5).

A procedure was also worked out for loading several incubation tubes sequentially and incubating them outside the system. This

*Acropor AN is an acrylonitrile-polyvinylchloride membrane reinforced with nylon.



SCALE : 4/1

DASH NO	H ±.005	Θ ±0°30'
-1	.185	90°0'
-3	.270	60°0°

MATERIAL : KEL-F

Figure 4

FILTER INSERTS

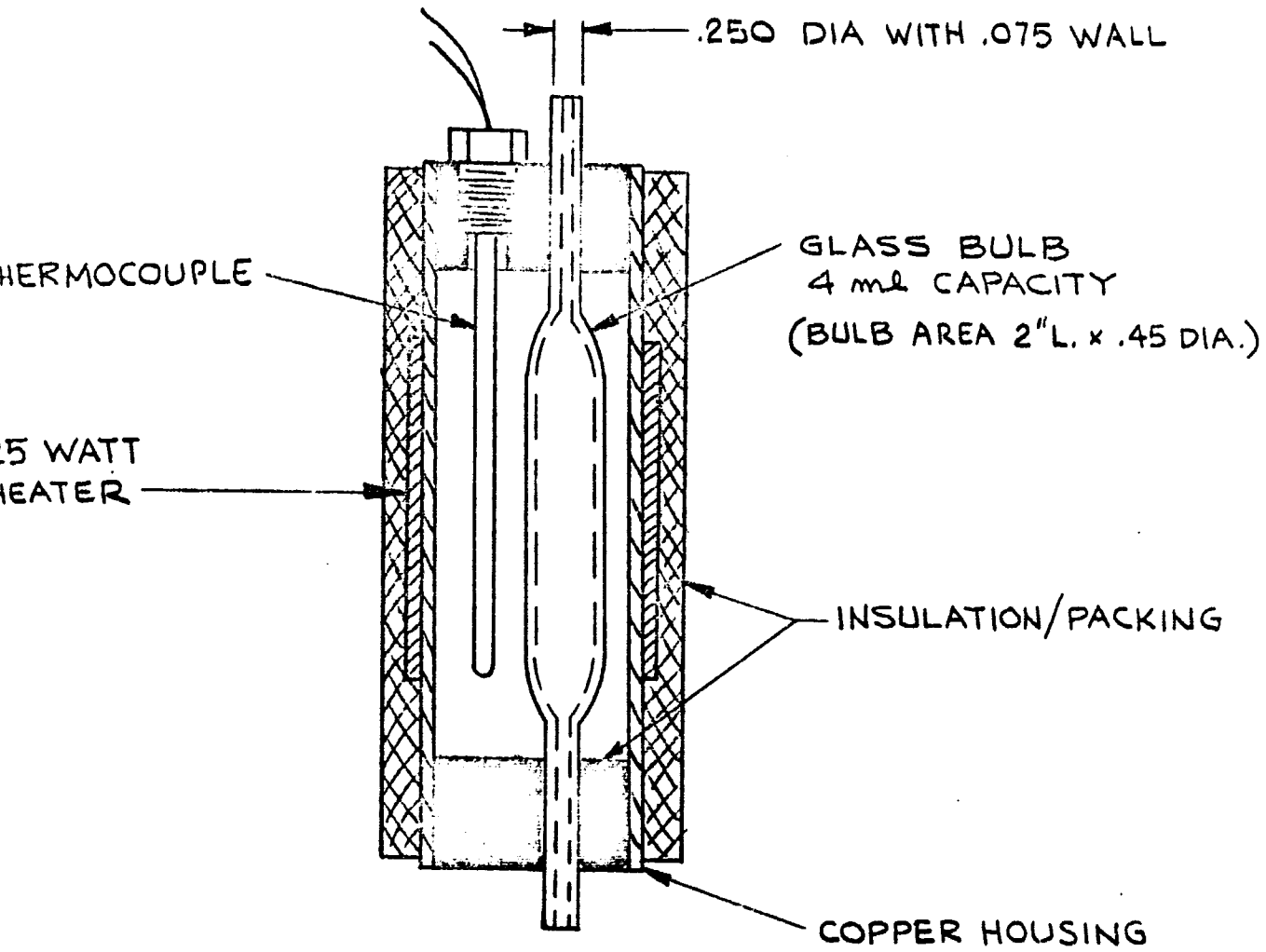


FIG. 5

INCUBATOR

increased the amount of data attainable within a single working day.

3.1.2.4 REACTOR CELL

Whereas dual reactor cells were utilized originally for independent monitoring of total and viable cell counts, only a single reactor cell of the type shown in Figure 6 is required in the final version of the cell monitor.

The reactor cell is fabricated of KEL-F/glass fittings and glass tubing. A split clamp assembly (Figure 6) serves as a mount to secure the reactor cell into the cell housing and to rigidly fix the spacing between the cell and the photomultiplier tube*.

3.1.2.5 READOUT SYSTEM

An EMI 9635B photomultiplier tube (PMT) is used as the light sensor. This tube is a two inch diameter end-on type with more than adequate sensitivity at 4300A^{o} (approx. peak emission of luminol chemiliminescence). Its equivalent noise input at peak wavelength is 1×10^{-16} watts, significantly lower than the 10^{-14} watt difference in light emission that we are trying to detect.

The signal output of the PMT may be plotted either as a direct (analog) or an integrated signal on a strip chart recorder.

3.1.2.6 REAGENT SYSTEM

The present composition of the reagents employed in the cell monitor is the following:

1. Luminol-H_2O_2

Whereas the luminol and hydrogen peroxide solution are stable for at least several months at ambient storage, the mixture of these two exhibits a significant loss in sensitivity after only 6 hours. Consequently these two reagents are mixed just prior to use by passing through a mixing coil with a 1/2 hour holdup time. Studies indicated that maximum sensitivity for this mixture is achieved at about 1/2 hour after mixing. Air bubbles are introduced into the luminol-H_2O_2 mixture at a uniform rate just prior to entering the reactor. The air bubbles serve to stabilize the reagent baseline by ensuring adequate mixing with the bacterial suspension.

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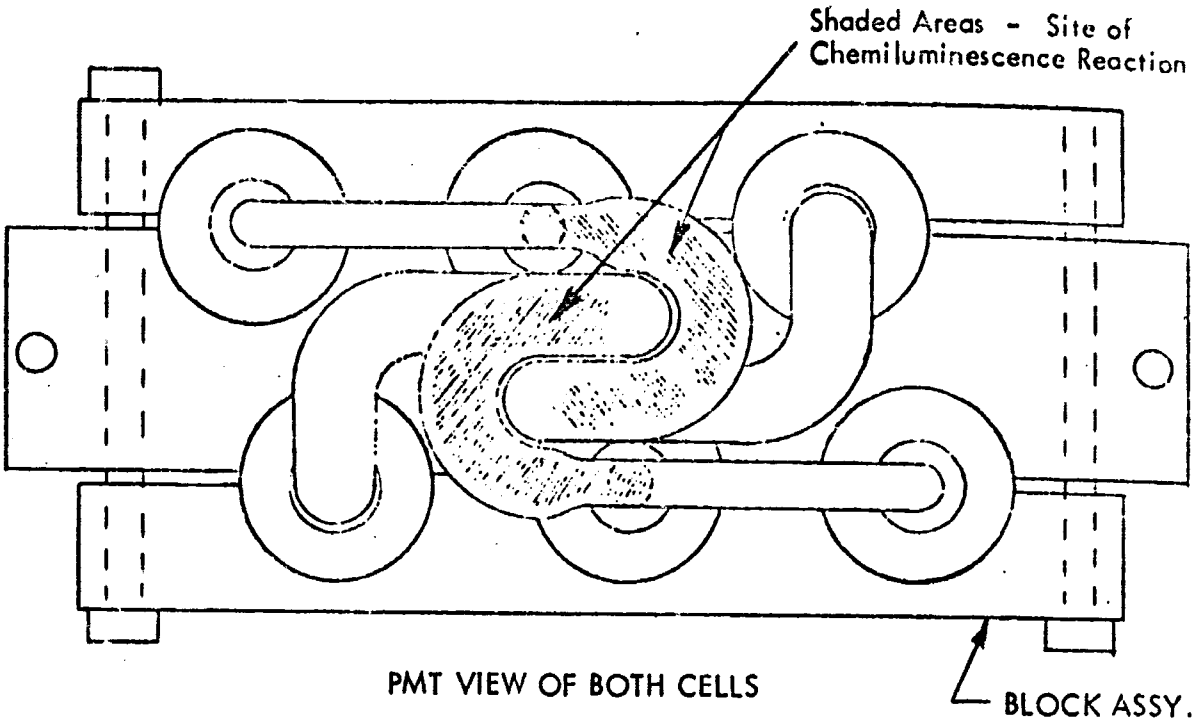
*A spacing of 0.010" is used between facings.



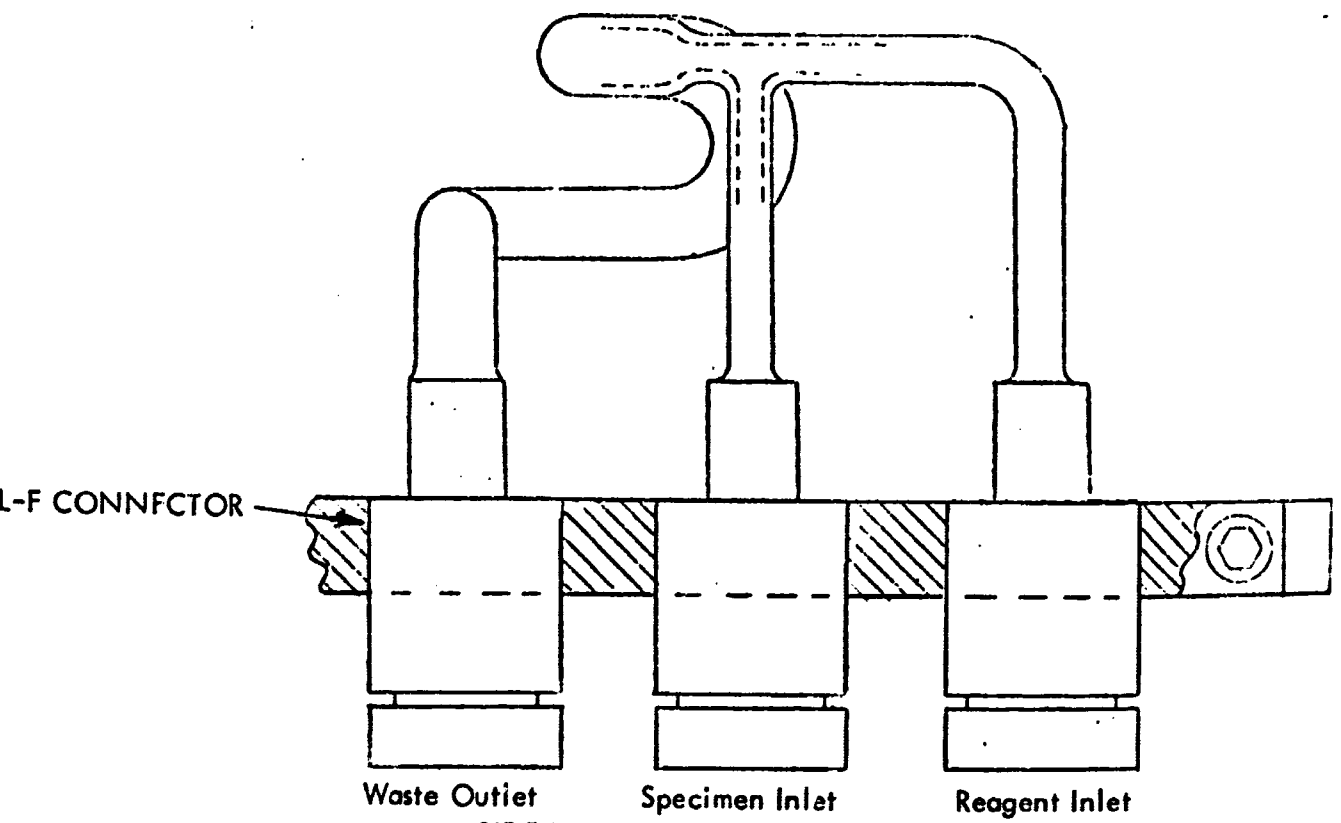


Figure 6

DUAL REACTOR CELL

2. Luminol Stock Solution

Luminol (Aldrich)*	1.00 grams
Disodium Ethylenedinitrilotetracetate (Baker Analyzed)	15.00 grams
Sodium Hydroxide (Baker Analyzed)	60.00 grams

Made up to one liter with glass-distilled Millipore filtered water.

Dilute 1:1 with glass-distilled, filtered H_2O just prior to use.

3. Hydrogen Peroxide Solution

3% H_2O_2 (Baker Analyzed) diluted to 1% with glass-distilled water just prior to use.

Other reagents which are utilized are as follows:

1. 8 M Urea

As a bactericide for flushing the system.

2. 2 M Urea

For backwashing bacteria off the final filter.

3. Dextrose Broth

For backwashing off the first concentrating filter and as an incubation medium in the viable cycle.

The preparation of these reagents is given in Appendix B.

3.2 LABORATORY STUDIES

The laboratory studies were designed to evaluate the operational performance of the cell monitor and to investigate a number of parameters for improving the detection threshold for the incubated and unincubated cycles. The results obtained are described below.

3.2.1 UNINCUBATED CYCLE (For monitoring of viable + non-viable cells)

3.2.2.1 CONTINUOUS OPERATIONAL MODE

In order to obtain a baseline value for the system, the sensitivity attainable without prior concentration of the water sample was first examined. In this mode of operation, the bacterial suspension was pumped directly into the reactor (at 1 ml/min) for reaction with the luminol-hydrogen peroxide reagent. This procedure could be used

*Dissolve NaOH and allow to cool before adding EDTA and luminol

for continuous monitoring of relatively high bacterial levels where soluble background interference was minimal. The results obtained with E. coli and two strains of S. marcescens are shown below. The effect of not introducing air bubbles into the luminol-hydrogen peroxide (Premix) reagent is also shown. PMT voltage was at 1210 v.

Organism Cells/ml*	Net Chemiluminescence** Signal, volts
With Air in Premix	
5×10^3 E. coli	0.2
1×10^4 E. coli	0.5
1×10^4 S. marcescens (NSMI)	0.4
1×10^4 S. marcescens	0.4
1×10^4 S. marcescens	0.5
1×10^4 S. marcescens (PP)	0.4
1×10^4 S. marcescens	0.5
Without Air in Premix	
5×10^5 E. coli	no response

These results dramatically illustrate the necessity for introducing air bubbles (at a uniform rate) into the reactor to enhance the sensitivity of the system. The air bubbles serve to stabilize an otherwise fluctuating baseline by facilitating mixing of the liquid streams at these low (non-turbulent) flow rates.

Regarding a net signal of 0.2 volts as significant in this mode of operation, these data would indicate that in the absence of an excessive soluble background, 5×10^3 - 1×10^4 cells/ml could be detected in a

*Flowing into reactor at 1 ml/min.

**Signal after subtraction of the reagent baseline. The signals shown in this final report are one-half in value to those reported in monthly reports. This factor of 1/2 was applied to all the data to compensate for an error in summing the resistance values of the electronic circuit used for processing the signal output of the PMT in the NASA breadboard unit.

continuously flowing stream without prior concentration of the sample.

Data obtained later in the program on reclaimed water from the MACDAC test series in Huntington Beach indicated that in most cases, the soluble background interference was too high for this mode of operation. Concentration by filtration and washing of the organisms were prerequisites for obtaining meaningful data in these instances.

3.2.1.2 INTERMITTENT OPERATIONAL MODE (UNINCUBATED CYCLE)

The best sensitivity for total (viable + non-viable) cells was obtained using the protocol outlined in Table 1. For a 100 ml sample, the detection thresholds for E. coli and S. marcescens were found to be 75 and 150 cells/ml, respectively.

The protocol shown in Table 1 actually passed through three stages of development; namely,

Stage 1 — Sample is processed through a single cycle of concentration, washing, and backwashing with 2M urea prior to reaction with reagent (Table 3).

Stage 2 — Sample passes through a double cycle with an intermediate step in which the backwashed organisms off the first filter are mixed with nutrient broth (Table 4).

Stage 3 — Similar to Stage 2 except that 2M urea is replaced by dextrose broth in the wash and backwash steps of the first cycle (Table 1).

Stage 1, referred to as the single cycle, required 56 minutes. to complete compared to the sequence in Stage 2, which required 48-73 minutes.* The latter procedure was investigated since it was felt that it would provide a more realistic control or baseline value for the viable cycle which followed. The detection threshold for Stage 1 and Stage 2 procedures were shown to be the same. However, better recoveries and higher sensitivities (toward total and viable cells) were achieved when 2M urea was replaced by dextrose broth in the first wash and backwash cycle (Stage 3).

---

*The lower value for Stage 2 was at a flow of 4 ml/min. to concentrate the sample; the higher value was at a flow of 2 ml/min.

Table 3

OPERATIONAL SEQUENCE FOR OBTAINING TOTAL CELL COUNT
(Unincubated Sample, Stage 1)

Operation	Flow Rate	Flow Duration
1. Concentration of Sample	2 ml/min.	50 min. *
2. Wash with 2M Urea	1 ml/min.	3 min.
3. Backwash with 2M Urea	1 ml/min.	3 min.
4. Reagent Flow		
Luminol	0.11 ml/min.	Continuous
H_2O_2	0.24 ml/min.	Continuous
Air	0.35 ml/min.	Continuous

*For 100 ml sample.

Table 4

TIMING SEQUENCE FOR OPERATION OF THE INCUBATED OR UNINCUBATED SAMPLE

(Stage 2)

Operation		Flow Rate	Flow Duration
1.	Concentration of Sample	2 or 4 ml/min.	25 or 50 min. *
2.	Wash (W_1) with 2M Urea (Nutrient on)	1 ml/min. (1 ml/min.)	2 min. (1 min.)
3.	Backwash (BW_1) with 2M Urea (Nutrient on)	1 ml/min. (1 ml/min.)	1 min. (1 min.)
4.	Incubate** Sample & Nutrient (Dextrose Broth) (1:1 mixture)	-	-
5.	Wash (W_2) with 2M Urea	1 ml/min.	10 min.
6.	Backwash (BW_2) with 2M Urea	1 ml/min.	5 min.
7.	Bactericide (8M Urea)	1 ml/min.	5 min.
8.	Reagent Flow		
	Luminol	0.11 ml/min.	Continuous
	H_2O_2	0.24 ml/min.	Continuous
	Air	0.35 ml/min.	Continuous

*For 100 ml sample (2 ml/min. used with 13 mm diameter filter; 4 ml/min. with 25 mm diameter filter).

**Incubation step omitted for obtaining total cell count.

3.2.1.2.1. PROTOCOL DEVELOPMENT STUDIES (STAGE 1)

Urea Vs. Water as a Backwashing Medium

Tests were conducted to determine which wash and backwash fluids could be used to provide for maximum recovery of organisms concentrated on a filter. Since this same medium would be used to recover organisms for subsequent incubation in the viable cycle, it was also important that the fluids selected did not interfere with bacterial growth.

The data obtained on comparing various concertrations of urea with water is summarized in Table 5. For this evaluation, 10 ml of a bacterial suspension containing 5×10^3 E. coli/ml were concentrated at a liquid flow of 2 ml/min. on a 13 mm diameter Acropor AN filter* (0.22 μ pore size), washed and then backwashed (1 ml/min. 3 min.) see Table 3) with either 2, 4, or 8M urea followed by reaction with luminol-H_2O_2 reagent.

The results of several runs made on different days are summarized in Table 5. Data obtained with water as a wash and backwash fluid are shown for comparison.

The results indicate that although 8M urea is superior to water, it is not as effective as either 2 or 4M urea. The difference between 2 and 4M urea is insignificant with 2M urea being prefered as it appears to produce more consistent results. The lower and more erratic recoveries obtained at the higher urea concentrations are probably due to a stickiness of the bacterial protein produced by excessive contact with urea in the wash step. While its properties as a protein denaturant render urea an effective backwashing medium for recovery of bacteria off a filter, its effectiveness is reduced if it is also used in the wash step. For maximum recovery where viability of backwashed organisms is not a requirement, the ideal sequence following concentration would be a water wash to remove soluble interfering components, followed by a 4 or 8 M urea backwash. While this sequence can be conveniently carried out with a bacterial detection device developed for the Army employing an indexing filter concentrator**, it is not readily performed on the present single filter unit. In the latter case, the wash and backwash fluids have to be the same. Data obtained with the Army chemiluminescent unit which illustrate the greater effectiveness of the more concentrated urea solutions are shown

* Gelman filter

** Exploratory Development of an Improved Chemiluminescence Detection System, Aerojet-General Report 1311FR-1, Sept., 1969, Contract DAAA 15-69-C-0065 for Dept. of the Army, Fort Detrick, Md.

Table 5

EVALUATION OF BACKWASHING MEDIA

Conditions

Sample concentration at 2 ml/min (10 ml sample)

Wash and backwash at 1 ml/min, 3 min duration

PMT voltage 1240

Wash and Backwash	E. coli cells/ml	Net Chemiluminescence Signal, volts
2M Urea	5×10^3	1.5
	"	2.5
	"	1.8
	"	1.5
	"	1.2
	"	1.5
	"	1.3
	"	1.5
		$\bar{X}$ = 1.6 (S = 0.4; $\frac{S}{\bar{X}}$ = 25%)
4M Urea	5×10^3	2.3
	"	1.7
	"	2.3
	"	1.7
	"	1.3
	"	1.7
	"	2.5
	"	0.8
	"	2.3
		$\bar{X}$ = 1.8 (S = 0.65/ $\frac{S}{\bar{X}}$ = 36%)
8M Urea	5×10^3	1.3
	"	1.3
	"	1.3
		$\bar{X}$ = 1.3 (S = 0)
Water	6×10^3	0.0
	"	0.8
	"	0.5
	"	0.0
		$\bar{X}$ 0.32 (S = 0.39; $\frac{S}{\bar{X}}$ = 122%)

$\bar{X}$ = mean, S = standard deviation, $\frac{S}{\bar{X}}$ = coefficient of variation

below. In this experiment, E. coli at 1.5×10^4 cells/ml was collected for 1/2 minute intervals (at a liquid flow of 1 ml/min.), washed with water (1 ml/min. for 1 min.) and then backwashed with aqueous urea at the concentrations indicated below. The signals obtained were as follows:

	8M Urea	4M Urea	2M Urea
Net Chemiluminescence Signal Volts	2	2	0

Based on data obtained with the single filter unit, 2M urea was initially selected as the backwashing fluid. Later studies (described under Protocol Development, Stage 3) indicated that dextrose broth was superior to 2M urea, not only in providing better bacterial recoveries, but in having less of an inhibiting effect on bacterial growth during the incubation cycle.

Effect of Velocity on Recovery Efficiency

One of the parameters affecting the recovery of organisms from a filter is the deposition velocity. The effect on recovery of depositing the bacteria at velocities of 1, 2, and 4 ml/min/cm^2 (effective deposition area of the 13 mm diameter filter is~0.8 cm^2) is shown by the data in Table 6. The results indicate that maximum recoveries in concentration of a 100 ml sample, as reflected by the increase in the chemiluminescence signal, appear to occur at flow rates of 1 ml/min. Although the sensitivities attainable at 1 ml/min. were about twice as good as those obtained at the higher flows, 2 ml/min. collection velocities were used in subsequent studies with the 13 mm diameter filter, because of the inordinately long time (i.e., 100 min.) required to concentrate a 100 ml sample.

Effect of PMT Voltage on Signal Strength

The effect of PMT voltage on signal strength was investigated with the objective of selecting the optimum voltage for maximum sensitivity. For these studies, the protocol shown in Table 3 was utilized. The results summarized in Table 7 indicate that the net signal increased in an approximately linear fashion with PMT voltage in the range of 1150 to 1340 volts. Subsequent studies were made at a setting of 1340 volts since the background noise was still not considered objectionable at this level.

Table 6

EFFECT OF FLOW RATE ON SENSITIVITY

Conditions

Wash and backwash with 2M urea at 1 ml/min (3 min duration)

PMT voltage at 1210

Flow Rate of E. coli Suspension	Sample Concentrated	Net Chemiluminescence Signal, volts
4 ml/min	100 ml (4×10^2/ml)	3.0
2 ml/min	100 ml (4×10^2/ml)	3.0
	100 ml (4×10^2/ml)	2.5
	10 ml (4×10^3/ml)	2.5
1 ml/min	100 ml (4×10^2/ml)	8.0
	100 ml (2×10^2/ml)	3.0
	100 ml (2×10^2/ml)	3.5

Table 7

EFFECT OF PMT VOLTAGE ON SIGNAL RESPONSE

PMT Voltage	Net Chemiluminescence Signal, volts	Bacterial Challenge
1150	1.5	1×10^4 E. coli/ml (10 ml*)
1210	2.0	"
1250	3.0	"
1300	3.8	"
1340	6.3, 4.5, 5.5	"
1340	2.5	4×10^3 E. coli/ml (10 ml*)

*Flow of liquid suspension at 2 ml/min.

Typical response curves for a water blank and a bacterial challenge at 1340 volts (direct or analog readout) are shown in 7A and 7B, respectively. The interval between A and B represents the time during which the sample (or sterile distilled water reference) is flowing through the concentration filter. In order to conserve reagent, the luminol-H_2O_2 flow has not as yet been started, so no significant signal is evident during this interval. At point B, a switch is activated which turns the sample stream off and starts a 2M urea wash. After 3 minutes, another switch initiates a backwash (point C) with 2M urea. The signal obtained on reacting the backwashed organisms with Premix reagent reaches its maximum in 30 to 45 seconds; about the same length of time is required to return to a baseline value. At point D, both the Premix and urea flows are turned off* and the system is readied for the next cycle.

The corresponding integrated responses for several water blanks and a bacterial challenge at the same level as that used in Figure 7B, are shown in Figures 8 and 9. The integration interval was 30 sec. (beginning at 20 sec. and ending at 50 sec. after initiation of the backwash). A comparison with the integrated values obtained over a different time interval (i.e., for 35 sec. between 15-50 sec. and 20-55 sec. respectively) did not show any improvement in the net luminescence (i.e., signal minus reagent blank). Thus,

Challenge: 4×10^3 *E. coli*/ml (10 ml sample); Gain 0-10, 0.01; PMT Voltage 1340

Integration Interval =	Signal Values in Arbitrary Scale Units		
	30 sec. (20-50 sec.)	35 sec. (15-50 sec.)	35 sec. (20-55 sec.)
Sample Signal (S) =	53.5, 52.5 (av. 53.0)	59.0	58.0
H_2O Blank (B) =	46, 46, 45 (av. 45.7)	54.5	54.5
Net Signal (S-B) =	7.3	4.5	3.5

With the present arrangement of the integration circuitry, the entire area (from zero baseline) under the curve is integrated. An alternate

*The spike appearing at point D is a transient pulse associated with the application of power to one of the solenoid valves. This was subsequently eliminated by isolating the solenoid from the electronic circuit.

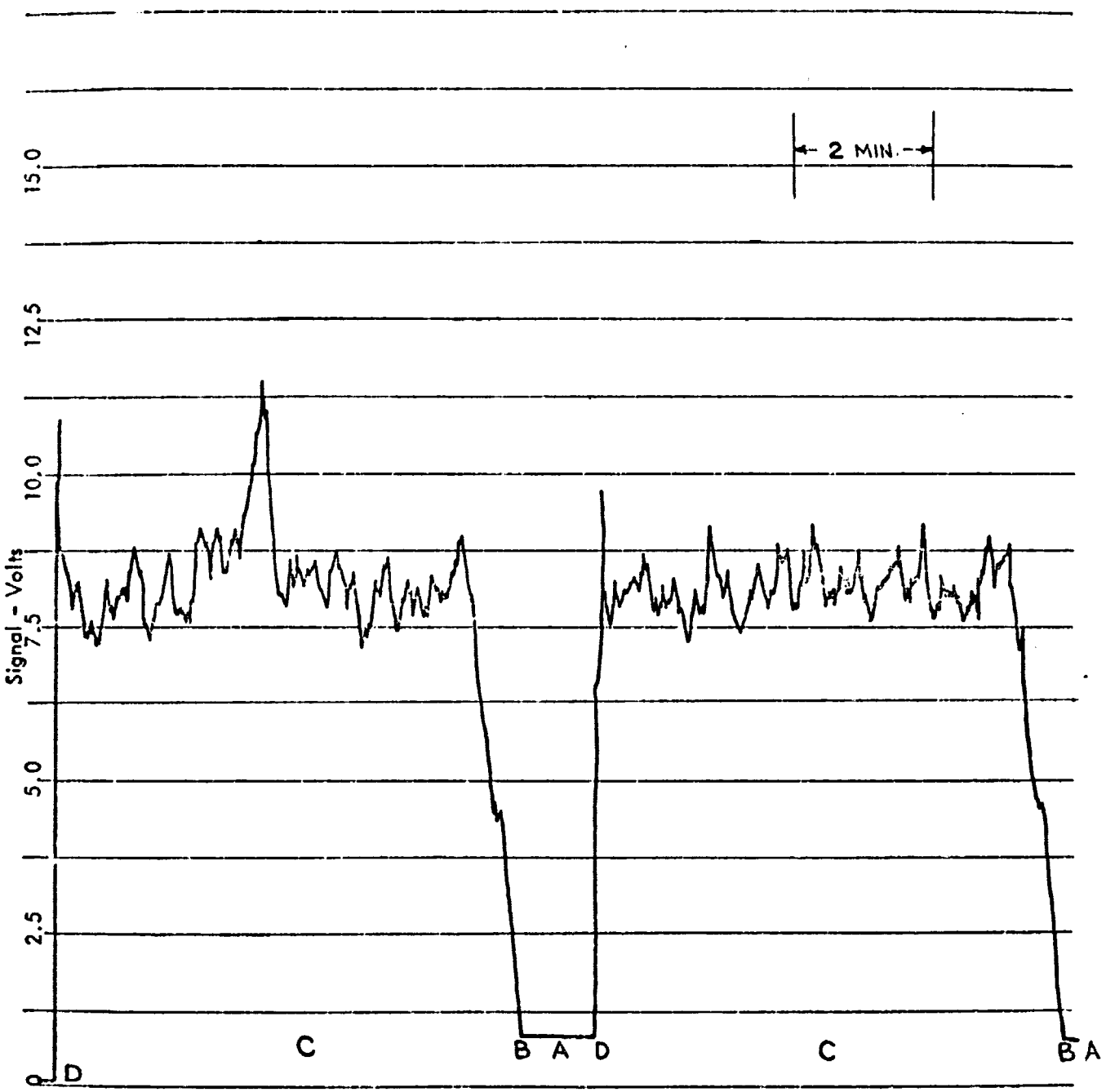


Figure 7A
SAMPLE RESPONSE

E. coli
4×10^3/ml
4×10^4 Total

Figure 7B
WATER BLANK RESPONSE

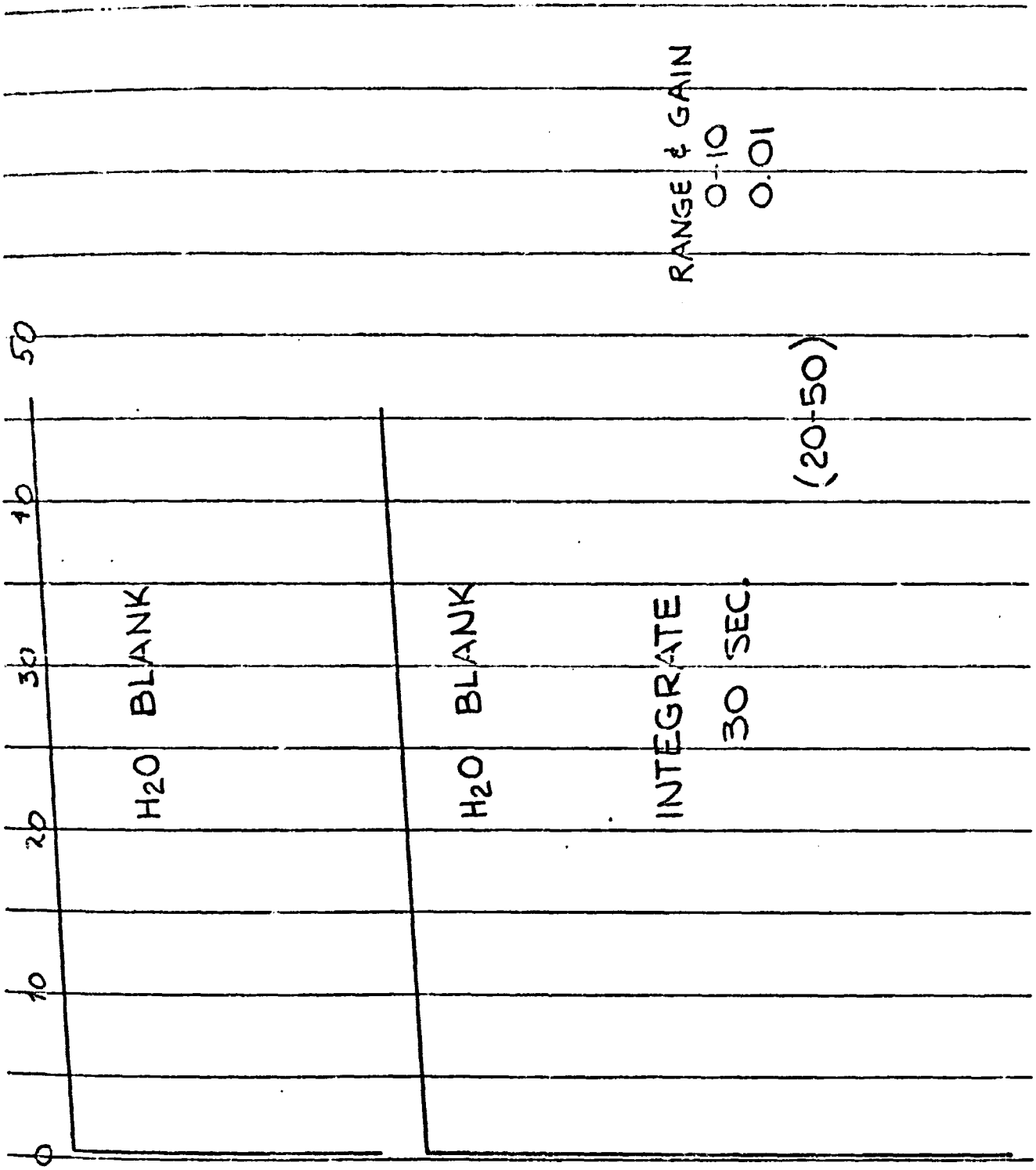


Figure 8
INTEGRATED SIGNAL - WATER BLANK

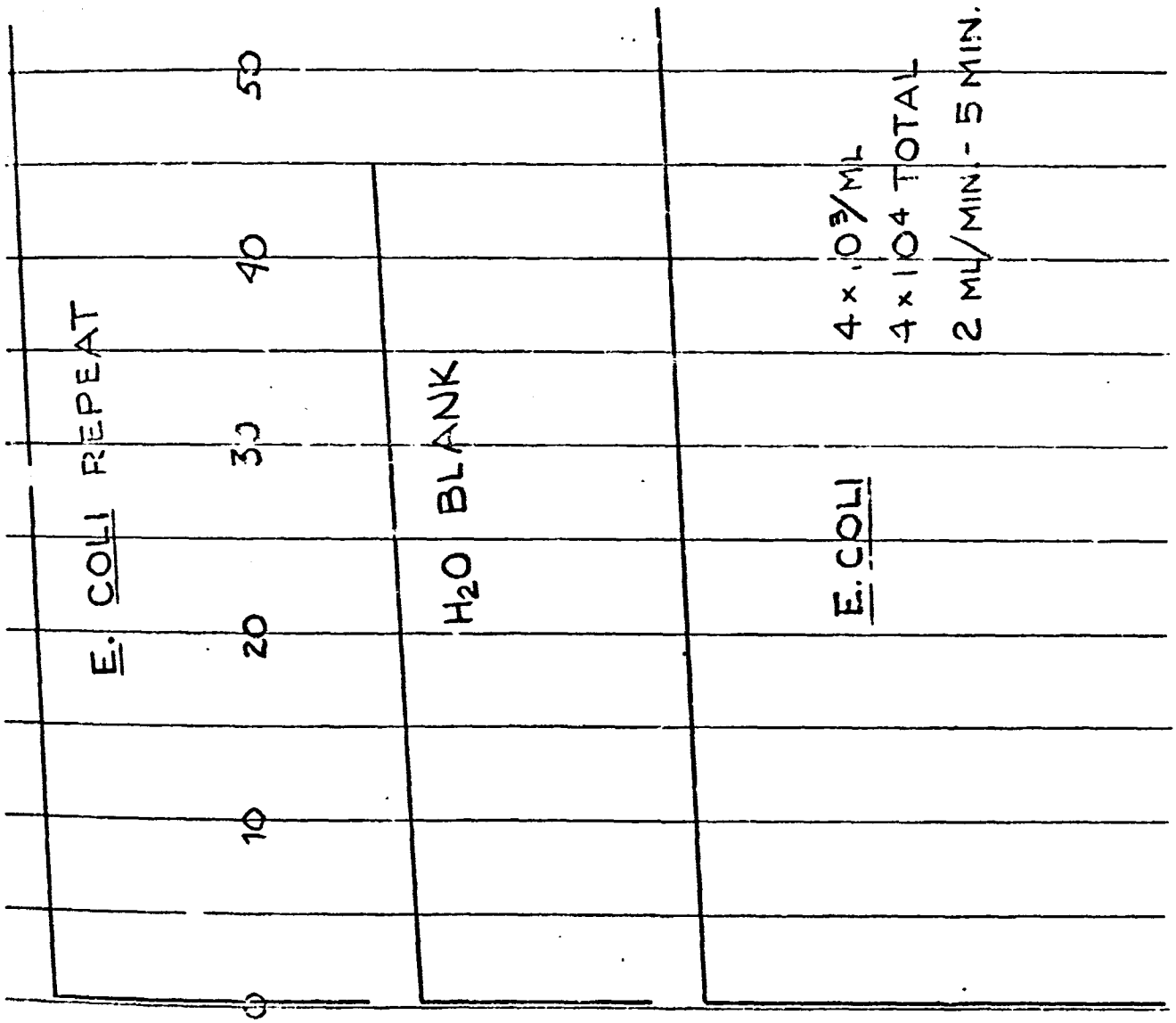


Figure 9
INTEGRATED SIGNAL - E. coli VS. WATER BLANK

arrangement would be one in which the reagent baseline voltage serves as a reference voltage which is subtracted from the integrated sample voltage. The only printout would be the net integrated signal appearing above the reagent baseline. This arrangement would permit use of a higher gain setting making smaller differences between sample and reagent more readily discernible. The feasibility of this approach remains to be investigated. In any event, since the present integration readout did not appear to offer any improvement in sensitivity over the direct or analog readout, the latter was used in all subsequent measurements.

Sensitivity of the Single Cycle Protocol

The sensitivity attainable with the single cycle protocol (Table 3) which had evolved as a result of the foregoing studies, was checked against E. coli and S. marcescens. The data summarized in Table 8 indicate that the sensitivity attainable on processing a 100 ml sample by this protocol was 200 cells/ml for E. coli and 400 cells/ml for S. marcescens*. The sensitivity ultimately reached with an improved protocol (Stage 3, Table 1) involving use of dextrose broth in place of 2M urea as a backwash was 75 and 150 cells/ml for E. coli and S. marcescens, respectively (see Section 3.2.1.2.3).

Bacterial Analysis of Reclaimed Water Samples

The feasibility of the established single cycle protocol (Table 3) for monitoring total cell content was evaluated against two regenerated water samples taken from the Space Station Simulator at McDonnell-Douglas in Huntington Beach on June 8th. A total cell count obtained by direct microscopic observation was used as a control. The results of these and other tests are described briefly below:

1) Sampling Procedure

a) Sample $608T_3$ - This water sample reclaimed

*Sensitivity obtained using a flow of 1 ml/min to concentrate the 100 ml sample instead of one standard 2 ml/min produced twice the signal (e.g., at 2 ml/min 200 E. coli/ml (100 ml sample) gave 1.3 volt net signal as opposed to 3.8 volts when the flow was 1 ml/min. A net signal of 1 volt is regarded as significant.

Table 8

SENSITIVITY DATA FOR E. coli and S. marcescens

(Unincubated Cycle)

Conditions: Sample concentration at 2 ml/min
PMT voltage 1340

Sample	Net Chemiluminescence volts
E. coli	
2×10^2/ml (100 ml)	1.3
4×10^2/ml (100 ml)	2.3
1×10^4/ml (10 ml)	6.3
1×10^4/ml (10 ml)	4.5
1×10^4/ml (10 ml)	5.5
1×10^4/ml (10 ml)	6.7
1×10^4/ml (10 ml)	5.0
	$\bar{X}$ = 5.6
S. marcescens	
4×10^2/ml (100 ml)	1.0

from urine by the Wick Evaporation Process* was taken at about 11:30 a.m. June 8th from water sampling port of heated storage tank T_3, located in the Space Simulator chamber. The sample was drawn by opening the sample valve and collected in sterile polyethylene containers with snap-on polyethylene lids.

b) Sample $608T_4C$ - This water sample, reclaimed by the Vapor Pyrolysis Process** was taken at about 11:45 a.m. June 8th from an aliquot contained in stainless steel pitchers (non-sterile) with lids, which had presumably been collected from T_4 storage tank of the Space Simulator that morning and kept at ambient. Sampled water was placed in sterile polyethylene containers with snap-on lids.

c) Both water samples above were maintained at ambient in transit and analysis initiated about 2 hours after sampling.

2) Tests Conducted on Water Samples at Aerojet

a) Viable Count - Standard pour plate method utilizing tryptone glucose extract agar was used. Incubation was for 4 days at 37°C.

b) Total Viable and Non-Viable Bacteria (two methods)

1 By direct microscopic count (at 2000X) of one ml samples concentrated by filtration through 0.22 μ Millipore (4 mm diameter filtering area) and staining with an Aerojet proprietary protein specific dye.

2 Estimation of count by comparing the net chemiluminescence signal of the sample to the signal obtained for an E. coli suspension of known concentration and handled the same way. The method entailed concentration of a 10 or 100 ml water sample (at 2 ml/min) by filtration on a 13 mm diameter 0.45 μ Acropor filter, washing with 2M urea (at 1 ml/min) to remove interfering components and then backwashing with 2M urea (1 ml/min) for reaction with luminol-H_2O_2 reagent (latter at 0.35 ml/min + 0.35 ml/min of air to facilitate mixing). Sterile distilled water similarly processed, was used as a control blank.

*Wick Evaporation Process - includes evaporation from a Wick evaporator, filtration and passage of the condensate through a series of activated charcoal and resin columns to remove the bulk of organic and inorganic materials. Reclaimed water was stored in heated storage tanks (165°F) for at least 5-6 hours before use.

**Vapor Pyrolysis (VDVF) Process - includes low pressure distillation followed by passage of the vapor through a heated (280-300°F) catalyst bed. The condensate is stored in holding tanks at 165°F for 5-6 hours before use. Passage through the activated charcoal and resin columns can be included, if required.

c) Soluble Signal - The chemiluminescence signal obtained by reacting the filtrate from the water sample (passing through the 0.45 μ Acropor) with luminol-H_2O_2 reagent.

3) Results

The results summarized in Table 9 indicate the following:

a) Viable Count: The viable count obtained at Aerojet for T_3 probably represents a minimum number (one ml rather than 0.1 ml aliquots were used for plating since it was assumed the counts would not exceed 10 cells/ml). The viable counts obtained by Aerojet do not agree with those obtained by McDonnell-Douglas; the actual values of the latter are to be made available at the conclusion of the 90-day test series by McDonnell-Douglas in their report.

b) Total Count: The total number of viable + non-viable bacteria estimated by chemiluminescence should be regarded as approximate only in view of the fact that the porphyrin content of different bacterial species vary. The number obtained by this method, however, appears to be consistent with that obtained by direct microscopic count, being of the same order of magnitude.

The absence of any significant chemiluminescence signal observed with a 100 ml sample of T_4C indicates that the number of bacteria present is below the detection limit, which for E. coli would be 200 cells/ml. Accepting the direct microscopic count as valid, failure to observe a signal in the present instance may be due to 1) the porphyrin content of the bacterial species present is inherently less than that of E. coli or 2) leaching of bacterial porphyrins due to the stress of high temperature cycling in the reclamation process. The predominant organism appearing in a later series of water samples obtained from the Space Simulator Station was identified as belonging to the Achromobacter group (see Section 3.2.1.2.3).

c) Soluble Signal - The high soluble signals observed with both water samples could be due to the presence of soluble porphyrins.

Actual traces obtained for sample $608T_3$ and a sterile distilled water control are shown in Figures 10 through 13. These traces were obtained by concentrating 10 ml aliquots (at 2 ml/min flow) of sample or sterile distilled water control. No significant net response was obtained on concentrating either 10 or 100 ml aliquots

Table 9

RESULTS OF ANALYSIS BY AEROJET OF RECLAIMED WATER SAMPLES

	Sample $608T_3$	Sample $608T_4C$
Viable Plate Count	500+/ml	~ 0/ml
Total Viable + Non-Viable		
a. Direct Microscopic	4.7×10^4/ml	5.6×10^3/ml
b. Estimated by Chemiluminescence	~ 2×10^4/ml*	~ none**
Soluble Signal	Approximately 4 times greater than that of sterile water	Approximately 11 times greater than that of sterile distilled water

*Based on an average value of ~11 volts obtained for duplicate determinations (i.e., 9 and 12.5 volts respectively) on Sample $608T_3$ (10 ml samples). Approximately 2×10^4 E. coli/ml (10 ml sample size) are required to produce a signal of this size using the single cycle protocol.

**100 ml sample used.

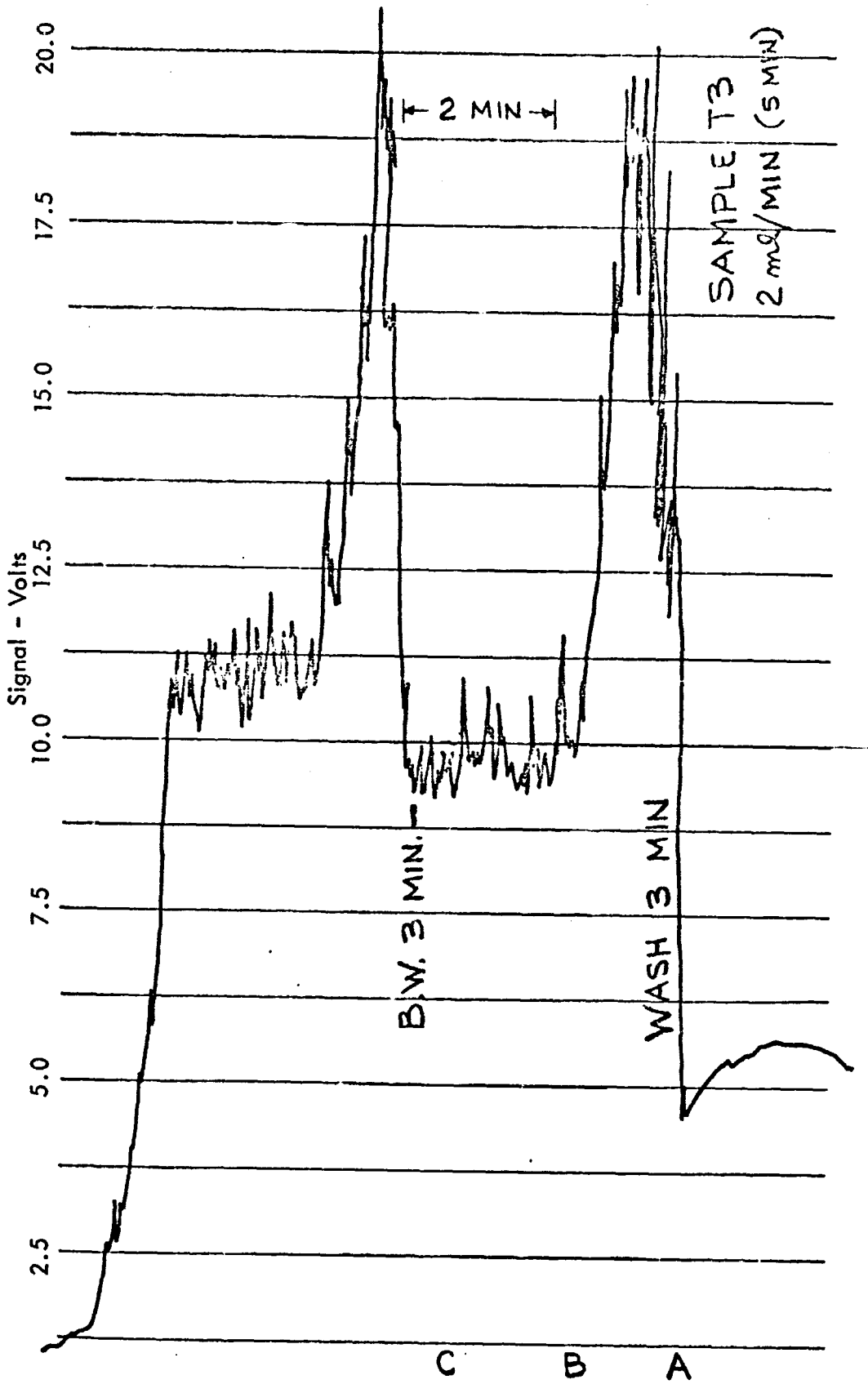


Figure 10
SAMPLE 608T3 RESPONSE (3 min. wash)

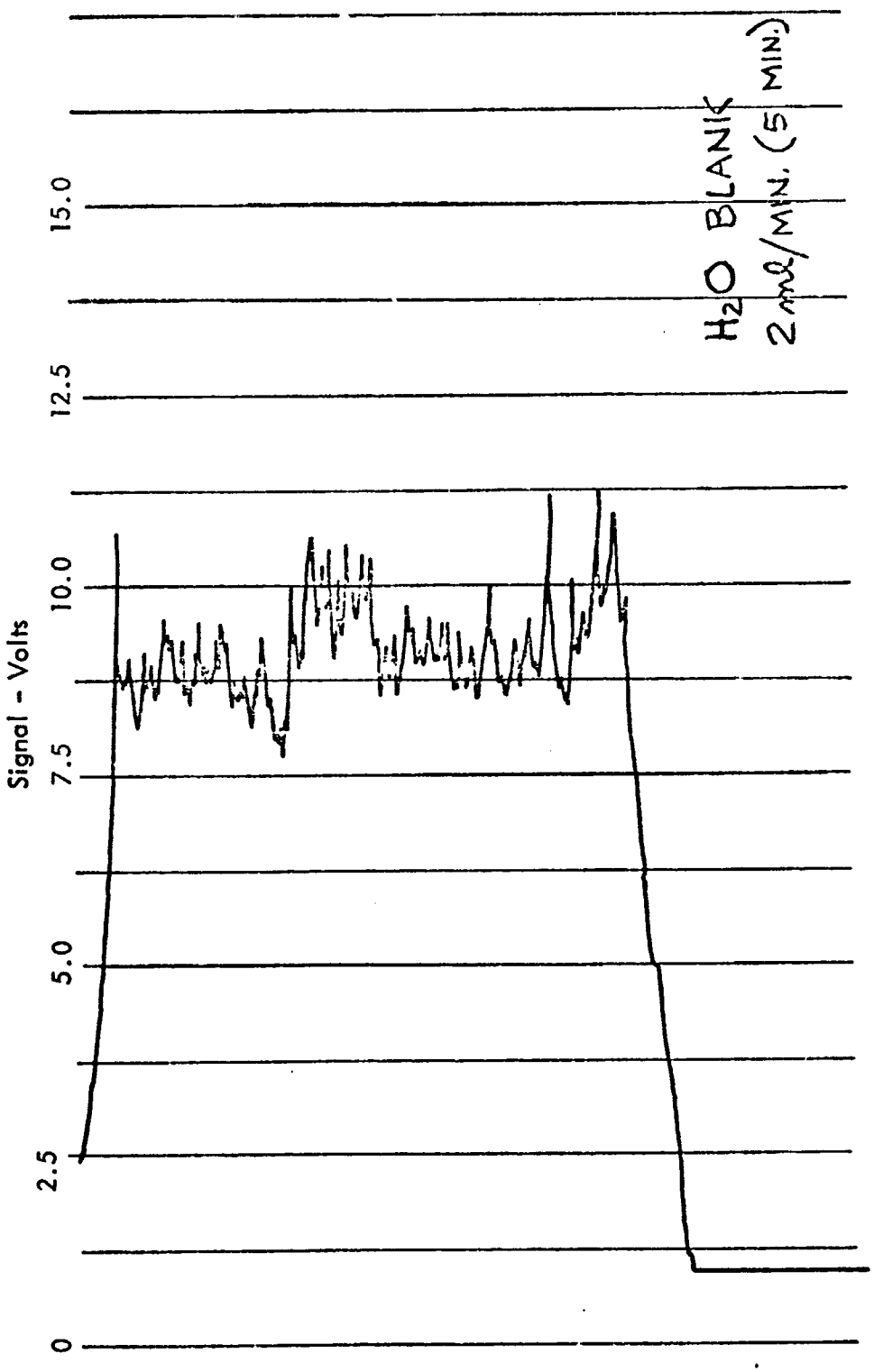


Figure 11

WATER BLANK RESPONSE (3 min. wash)

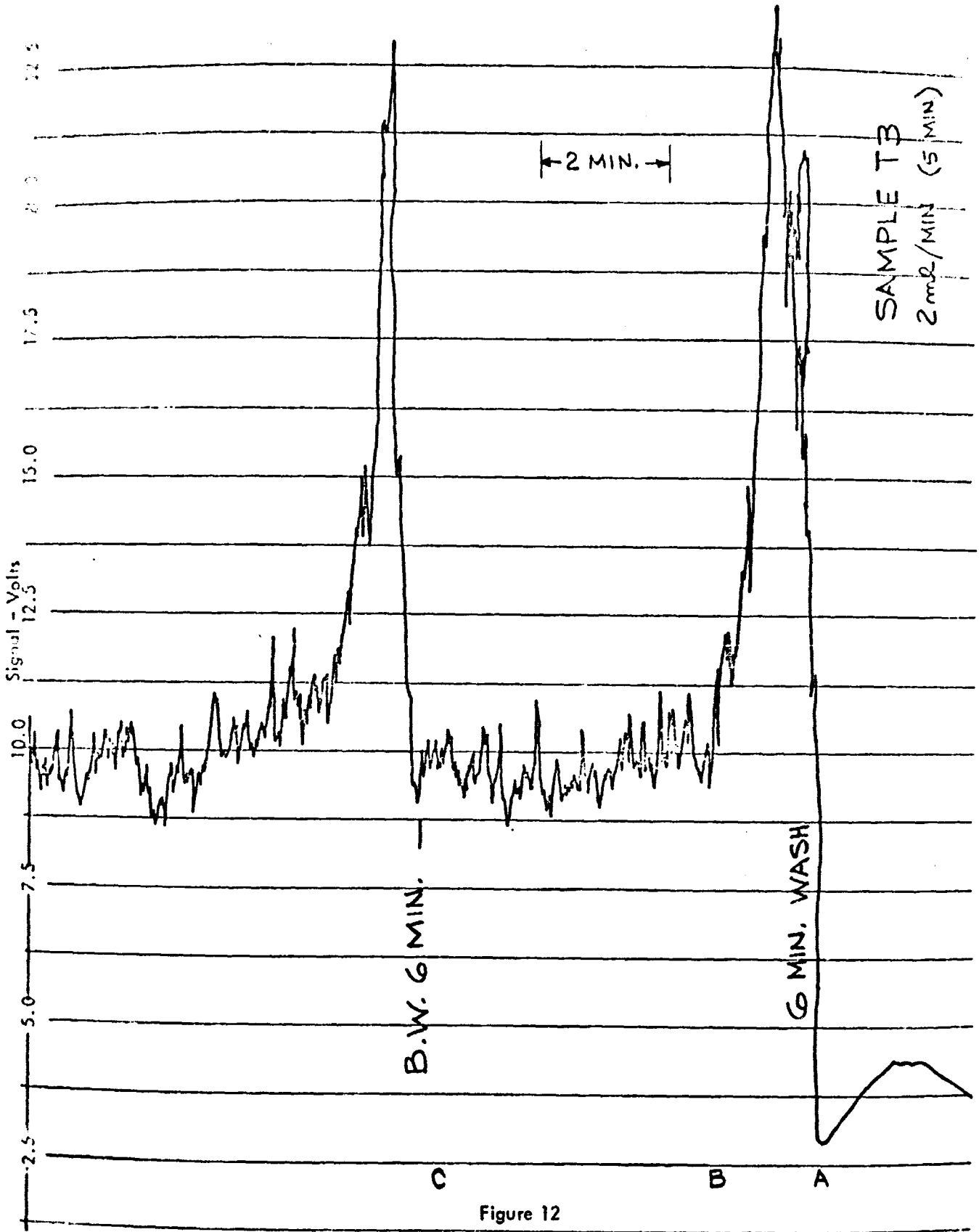


Figure 12

SAMPLE 608T3 RESPONSE (6 min. wash)

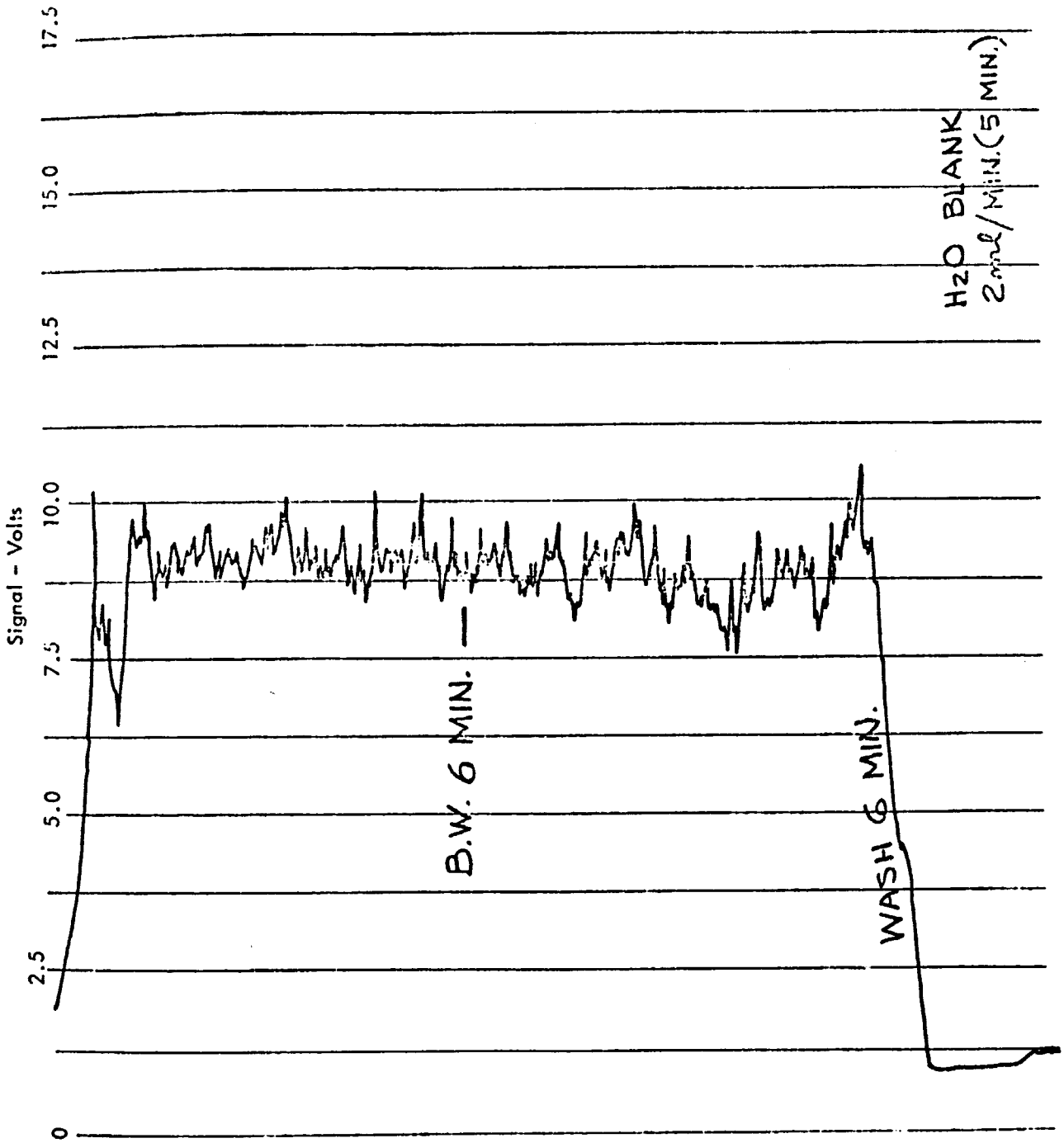


Figure 13

WATER BLANK RESPONSE (6 min. wash)

of sample $608T_4C$.

Referring to Figures 10 and 12, the interval from A to B represents the soluble signal obtained on washing the concentrated sample on the filter with 2M urea for 3 or 6 minutes. It is apparent that a 3 minute wash is sufficient to bring the signal back to baseline. Backwashing occurs at point C with the subsequent signal of 18 and 25 volts respectively being due to reaction of backwashed organisms with Premix reagent in the reactor. Traces obtained for the sterile water controls are shown for comparison.

High water solubles were also observed in reclaimed water samples analyzed at the conclusion of the test series at McDonnell-Douglas on September 11 (see Section 3.2.1.2.3). Samples with high soluble signals in that series generally exhibited relatively low total cell counts as well as low chemiluminescence (bacterial) signals.

3.2.1.2.2 PROTOCOL DEVELOPMENT STUDIES (STAGE 2)

The sample processing sequence evolved during this stage of development is summaried in Table 4. It consisted of two cycles of concentration, washing and backwashing (2M urea) with an intermediate step in which the organisms backwashed off the first filter are mixed with nutrient in the incubator. Since this procedure is identical to that used for processing the incubated sample in the viable cycle, it should provide a more meaningful baseline signal for the incubated sample.

A typical trace obtained for an unincubated sample containing added bacteria is shown in Figure 14*. The sample consisted of 10 ml of E. coli suspension (1×10^4 cells/ml) that had been treated as described in Table 4. Figure 15 is a water control** similarly processed.

Referring to these figures, the indicated intervals have the following significance:

A to B: sample is being concentrated on Filter no. 1 (Figure 1).

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*In actual practice, it would not be necessary to monitor the entire processing sequence but only that portion dealing with reaction of the backwashed organisms with reagent.

**Sterile distilled water used for preparing the E. coli suspension.

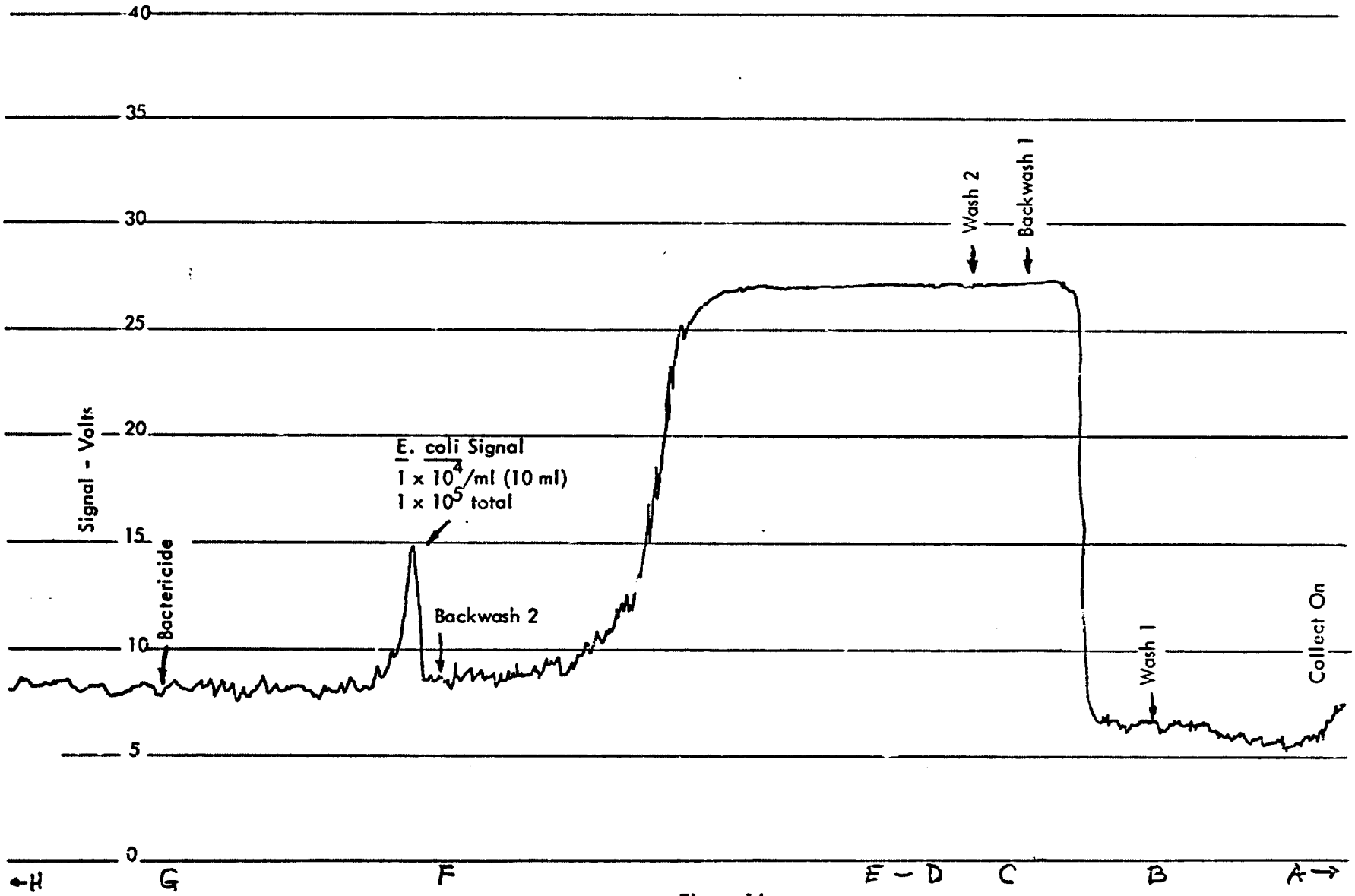

Figure 14
UNINCUBATED E. coli SAMPLE RESPONSE

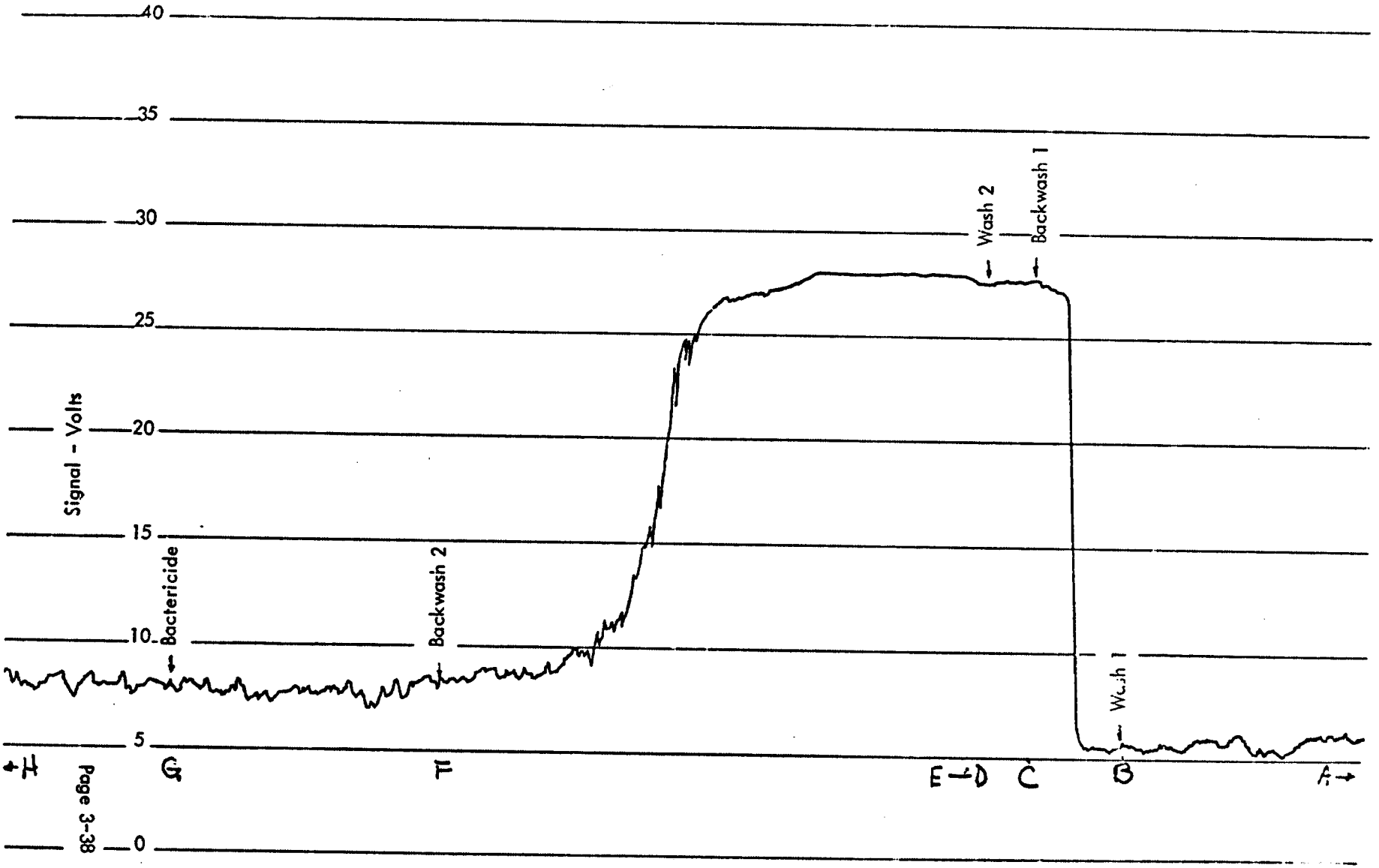

Figure 15
UNINCUBATED WATER BLANK RESPONSE (10 ml)

Page 3-38

B to C: concentrated bacteria are washed with 2M urea, the nutrient is also flowing through the system at this time and the large increase in signal is due to reaction of reagent with nutrient (probably due to porphyrins in beef extract, one of the components of Dextrose broth).

C to D: bacteria are backwashed off filter and enter incubator mixed with nutrient broth.

D to E: incubation period (zero for water control); chart drive off during incubation.

E to F: sample is concentrated on Filter No. 2 (Figure 1) and washed free of nutrient with 2M urea (W_2), as indicated by a decrease in signal to a stable baseline value.

F to G: bacteria are backwashed off Filter No. 2 with 2M urea and react with luminol-H_2O_2 reagent.

G to H: system is flushed with bactericide; 8M urea was substituted for Consan-20* (used in earlier studies) when it was found to be just as effective and possess the added advantages of being more easily flushed out of the system and more effective in removing residual protein.

Subtracting the signal obtained for the water control from the sample signal (interval F-G) gives a net signal of about 5.5 volts. Data for a number of runs performed on different days are shown in Table 10. The observed variations could be due to variations a) in recovery of bacteria off the filter, b) in the accuracy of the direct microscopic counts and c) in growing and harvesting the bacteria (16 hr cultures) under presumably identical conditions. In spite of these factors, the average value obtained by the protocol outlined in Table 4, is virtually the same ($\sim$ 6.4 volts for 1 x 10^4 E. coli/ml - 10 ml sample) as that obtained in the single cycle shown in Table 3 (which yielded an average net signal of 5.6 volts for the same number of organisms)**. Based on these results it appeared that the overall bacterial recoveries of the two subsystems were comparable, in spite of the fact that the more complex processing sequence contained two cycles of concentration, washing and backwashing. A direct microscopic count of the bacterial recoveries performed on each of the filters (data summarized in Table 11) indicated 58-66% off the first filter and essentially 100% off the second filter. The presence of

*Consan-20 (Consan Pacific Inc.) is a mixture of alkyl benzyl ammonium chlorides.

**See Table 8.

Table 10

SIGNAL RESPONSE FOR UNINCUBATED SAMPLES

(Stage 2 Protocol)

Conditions:

Sample size - 10 ml (concentrated at 2 ml/min)

PMT voltage - 1340

E. coli Concentration (Viable and non-viable)	Net Chemiluminescence Signal, Volts*
1×10^4/ml	6.0
1×10^4/ml	4.5
1×10^4/ml	3.5
1×10^4/ml	8.0
1×10^4/ml	5.5
1×10^4/ml	5.5
1×10^4/ml	7.0
1×10^4/ml	8.5 9.0 $\bar{X}$ = 6.4 ±2.1 (S**)
5×10^3/ml	4.0

*Values corrected for water blanks

**$\bar{X}$ = mean, S = standard deviation

Table 11

BACTERIAL RECOVERY EFFICIENCIES

1st Filter* (in duplicate)

	% of Recovery
Run No. 1	58
Run No. 2	66

2nd Filter (backwashed off 1st & 2nd filters)

Run No. 1	66
Run No. 2	66

*A 10 ml sample containing 6×10^4 cells/ml was collected on the first filter (0.8 cm^2 filtering area; liquid flow 2 ml/min) and then washed and backwashed with 2M urea using the standard procedure. The number of organisms contained in the first ml of backwash was determined by direct microscopic count of organisms stained with a protein specific dye.

nutrient on the surface of the second filter probably facilitated recovery of bacteria on backwashing.

Effect of Filter Size on Bacterial Recovery Efficiencies

The feasibility of employing a larger filter for concentrating the initial water sample was investigated. As shown earlier (Table 6), one of the factors affecting bacterial recoveries from a filter was the deposition velocity, with lower flow rates favoring a higher bacterial recovery. The maximum recovery on concentrating a 100 ml sample on a 13 mm diameter filter was shown to occur at a deposition velocity of about 1 ml/min/cm^2. Use of a larger filter would speed up the concentration step by permitting higher flow rates.

The recovery efficiencies of a 13 and 25 mm diameter filter were evaluated by comparing the signal obtained with a fixed bacterial challenge and the processing sequence in Table 4*. The results for a number of runs summarized in Table 12 indicate the following:

1) Based on the average values of a number of runs, the net signal is increased by approximately 100% on substitution of a 25 mm for a 13 mm diameter filter (at a constant flow rate of 2 ml/min).

2) For the 25 mm diameter filter, the signals at a 4 ml/min are comparable to those at 2 ml/min.; a slight if significant dropoff in the signal appears to occur at 6 ml/min flow.

The ability to operate at a liquid flow of 2 ml/min instead of the standard 2 ml/min reduced the sample concentration time in half (i.e., from 50 min to 25 min).

3.2.1.2.3 PROTOCOL DEVELOPMENT STUDIES (STAGE 3)

The major changes made in the operating procedure during this stage of development were 1) replacement of 2M urea by dextrose broth in the first wash and backwash steps and 2) modification of the reflector behind the reactor cells.

---

*Only the first concentrating filter (Figure 1) is interchanged (i.e., 25 or 13 mm); the second filter used for concentrating the bacterial suspension after incubation remains 13 mm.

Table 12

COMPARATIVE SIGNALS OBTAINED WITH 13 mm AND 25 mm DIAMETER FILTERS*

Flow Rate**	Net Signal, Volts	
	13 mm Dia. Filter	25 mm Dia. Filter
2 ml/min	6.0	9.5
	4.5	
	3.5	11.5
	3.0	
	5.5	13.0
	5.5	
	7.0	
	9.0	
	8.5	
	$\bar{X}$ = 6.4	$\bar{X}$ = 11.3
4 ml/min	-	12.0
		10.5
		11.5
		11.5
		$\bar{X}$ = 11.4
6 ml/min	-	7.5
		11.0
		$\bar{X}$ = 9.3

*10 ml sample containing 1 x 10^4 E. coli/ml, Stage 2 Protocol (Table 4) data obtained on different days.

**Flow rate used to concentrate sample.

While the first innovation did not alter signal response significantly, it did lead to a marked reduction in the incubation time of the viable cycle. Improving the design of the reflecting surface behind the reactor cell did increase signal response. The studies which led to the optimum procedure for total cell count shown in Table 1. are described briefly below.

Dextrose Broth vs 2M Urea as a Backwashing Medium

Based on studies to be described later, it became evident that urea, even at low concentrations (i.e., 0.5 Molar), inhibited the rate of bacterial growth. The feasibility of using Dextrose broth to replace 2M urea as a backwashing medium on the first filter was investigated by comparing the relative signals attained with a fixed bacterial challenge. The results shown in Table 13 indicate that comparable signals were obtained. Additional runs performed with 100 ml suspensions of E. coli and S. marcescens using the developed protocol shown in Table 1 indicated detection thresholds* of approximately 100 and 200 cells/ml, respectively (Table 14).

Bacterial Analysis of Reclaimed Water Samples from MACDAC**

The processing sequence for total cell count shown in Table 1 was evaluated against reclaimed water samples obtained from the Space Simulator Station at McDonnel'-Douglas in Huntington Beach. The details follow:

1) Sampling

Nine water samples were taken from the Space Station Simulator at McDonnell-Douglas (Huntington Beach) on conclusion of their test series on September 11. The principal method used for water reclamation was the vapor pyrolysis technique (i.e., low pressure distillation of urine followed by passage of the vapor distillate through a heated catalyst bed (ca 250-300°F) and storage of the effluent in heated holding tanks (T_1 and T_2) maintained at pasteurization temperature). After acceptance testing, the water was used to supply all the other taps (i.e., Hot, Cold, T_3, T_4, T_5, T_6 - all except wash water tap).

Sampling from Tanks T_1 and T_2 was through a hose

*One volt taken as a significant signal.

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Table 13

COMPARATIVE SIGNALS OBTAINED WITH 2M UREA AND DEXTROSE BROTH AS A BACKWASHING FLUID*

	Net Signal (volts)
2M Urea Wash* and Backwash	12.0
	10.5
	- - - - - - - - - - -**
	11.5
	11.5
	$\bar{X}$ = 11.4
Dextrose Broth Wash and Backwash	14.5
	13.0
	- - - - - - - - - -
	12.0
	10.5
	10.5
	12.5
	- - - - - - - - - -
	10.0
	$\bar{X}$ = 11.9

*10 ml water sample containing 1 x 10^4 E. coli/ml, 25 mm diam. first filter (Dextrose broth or 2M urea as backwash), 13 mm diam. second filter (2M urea as backwash); 4 ml/min flow used to concentrate sample; wash and backwash flows at 1 ml/min.

**Data separated by dashed line obtained on different days.

Table 14

DETECTION THRESHOLD FOR TOTAL COUNT (VIABLE + NON-VIABLE) OF *E. coli* AND *S. marcescens*

Organisms	Total Cells/ml*		S-N Net Signal, Volts
E. coli	50/ml		0.5
	100/ml		1.5
			1.5
			0.8
		$\bar{X}$ =	1.3
	200/ml		4.0
			4.0
		$\bar{X}$ =	4.0
S. marcescens	100/ml		0
			0.5
	200/ml		2.0
			2.0
			2.0
			3.5
			2.0
		$\bar{X}$ =	2.3

*100 ml sample; processed according to sequence shown in Table 1.

attached to spigots on each of the respective tanks. Sampling of all others was through a detachable metal pipe (ca 6" in length) with a quick snap-on connector at one end and a spigot on the other.

At the time of sampling, the reclamation unit had been off for several hours.

Water samples for testing at Aerojet were drawn into sterile polyethylene containers with snap-on polyethylene lids.

2) Tests Performed at Aerojet

a) Viable Counts: Standard pour plate method utilizing tryptone glucose agar was used. Incubation was for 4 days at 37°C.

b) Total Cell Count (Viable + Non-Viable) - 2 methods

1 By direct microscopic count (at 2000X) of one ml samples concentrated by filtration through a 0.22 μ Millipore (4 mm diameter filtering area) and stained with an Aerojet proprietary protein specific dye.

2 An estimation of the relative number of total cells was also made using the net chemiluminescence signal obtained in the water monitor. The method entailed concentration of a 10 or 100 ml water sample (at 4 ml/min) by filtration onto a 25 mm diameter 0.45 μ Acropor filter, washing and backwashing the concentrated organisms with nutrient (Dextrose) broth, and concentration of these organisms onto a second filter (13 mm), washing and backwashing with 2M urea followed by reaction with luminol-H_2O_2 reagent in the glass reactor. The total processing time was either 24 or 46 minutes depending on sample (10 or 100 ml) size. Sterile distilled water similarly processed, was used as a control blank.

c) Soluble Signal: The chemiluminescence signal obtained by reacting the filtrate from the water sample (passing through a 0.45 μ Acropor filter) with luminol-H_2O_2 reagent. This parameter was determined two ways - from the soluble signal taken from the strip chart recorder of the water monitor and by examining the filtrate in a cell monitor developed by Aerojet for commercial application.

The results are summarized in Table 15. The respective columns in this table contain the following information.

Column

1	sample designation
2	viable plate count
3	direct microscopic total cell count

Table 15

ANALYSIS DATA FOR MACDAC WATER SAMPLES

Sample	Viable Cells/ml	Total Cells/ml (Dir. Micro.)	Net Chem. Signal Volts*	Soluble Signal**
T5	$>10^3$(5)***	5.2×10^3	5	47 x
T3	$>10^3$(6)***	8.8×10^3	10	4.1 x
Hot	~0	1.0×10^4	5	46 x
Cold	$>10^3$(4)***	2.7×10^4	35	-
Wash	26	4.9×10^4	50	108 x
T4	$>10^3$(3)***	1.3×10^5	170	10.6 x
T1	~0	2.8×10^5	100	11.5 x
T2	$>10^3$(1)***	2.9×10^5	185	10.6 x
T6	$>10^3$(2)***	9.9×10^5	170	9.4 x

*Data normalized to a 100 ml sample.

**By analysis with Aerojet Cell Monitor of filtrate passing through 0.45 u filter (relative to that of distilled water, used as a control).

***Relative ranking according to numbers estimated visually.

NOTE: Since the water samples were taken approximately 10 hours after the system was shut down, and the pour plates were incubated 4 days rather than the standard 2 days, the bacterial counts were higher than those reported by the prime contractor (McDonnell-Douglas). It is expected that if the plates had been counted after 2 days incubation rather than 4 days, the viable counts would have agreed with those of McDonnell-Douglas.

Column

4 net chemiluminescence signal (data all normalized to a 100 ml water sample); a 1 volt signal would be considered significant.

5 soluble signal - numbers indicated were those obtained with the Aerojet Cell Monitor and represent the signal magnitude relative to that obtained with sterile distilled water, used as a control.

3) Test Results

a) The viable count as determined by the pour plate method exceeded 10^3 cells/ml in 7 of the 9 samples tested. An accurate count for these samples was not available, since undiluted 1 ml samples had been plated out on the assumption that the count would be < 10/ml. However the plates containing $> 10^3$/ml were reexamined and ranked according to their approximate number.

Achromobacter was one of the organisms isolated and identified as being present on these plates by an independent laboratory*.

In identifying these gram negative organisms the material sampled from the pour plates is streaked on MacConkey agar plates. This separates organisms that ferment lactose from those organisms that do not. The next step was to set up bio-chemical reactions with Kligers agar, Citrate agar and Indole media. The Kligers will differentiate organisms that do not ferment glucose from those that do and also determines production of gas and hydrogen sulfide. The other two media are for the determination of the utilization of citrate as carbon sources and the action on tryptone to produce indole.

The predominant organism appearing on all the pour plates used for determining viable count (Table 14) was both non-lactose and non-glucose fermenting. This will classify them as Pseudomonas or Achromobacter or organisms very similar to these two. Cytochrome oxidase strips are used to differentiate Pseudomonas species from other organisms giving similar bio-chemical reactions. Organisms sampled from each of the culture plates gave a negative result for this test and so were included in the Achromobacter group. Almost all of the organisms were slow growing.

b) The total (viable + non-viable) number of bacterial cells, determined by direct microscopic count, ranged from 10^3 to 10^6 cells/ml.

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*Reference, Laboratory, North Hollywood, Calif.

McDonnell-Douglas water samples analyzed earlier (Section 3.2.1.2.1) had also exhibited total cell counts ranging from $\sim 5 \times 10^3$/ml to 5×10^4/ml. If these numbers are representative of the current state of the art of water reclamation systems, the detection sensitivity requirements of < 10 cells/ml would appear unrealistic. The total cell count of potable tap water generally runs between 10^3-10^4 cells/ml.

c) The soluble signal (i.e., signal obtained on reacting filtrate with luminol-hydrogen peroxide reagent) for each of these samples was found to be high, ranging from 4- to 100-fold greater than that shown by freshly distilled water. The presence of a soluble fraction which produces a signal larger in most instances than that generated by the bacteria themselves, indicates why pre-washing of the bacteria, prior to reaction, is essential.

Evidence that this soluble fraction is due principally to porphyrins from lysed organisms is based on the following considerations:

a) If the samples are ranked in decreasing order for each of the parameters, the following is observed:

Chemiluminescence (Bacterial) Signal	T_2 >	T_6 = T_4 >	T_1 >	Wash >	Cold >	Hot =	T_5 >	T_3	
Viable Count	T_2 >	T_6 >	T_4 >	Cold >	T_5 >	T_3 >	Wash >	T_1 =	Hot
Total Count (Dir. Micro.)	T_6 >	T_2 >	T_1 >	T_4 >	Wash >	Cold >	Hot >	T_3 >	T_5
Soluble Signal									
from recorder chart	Wash >	Hot >	Cold >	T_5 >	T_1 >	T_2 >	T_4 >	T_6 >	T_3
by Aerojet Cell Monitor	Wash >	Hot >	T_5 >	T_1 >	T_2 =	T_4 >	T_6 >	T_3	

The ranking reveals that

(1) Except for minor differences, the chemiluminescence signal follows the same order as the total cell count. While not necessarily expected, the viable count also shows a similar trend (at least for the extreme cases such as T_2, T_6 and T_4).

(2) The fact that samples exhibiting a high soluble signal (i.e., Wash, Hot, Cold and T_5) have a low total cell count as well as a low chemiluminescence bacterial signal suggests that the solubles are produced by lysis

of the organism, rather than to chemical interference, or a leaching of the porphyrins. Cell lysis probably occurs on pasteurization or during the high temperature processing on reclamation.

Sample T_3 appears to be unique in possessing not only a low soluble but a relatively low total cell count and low chemiluminescence signal as well. This would suggest that the bacterial count of this sample was at a relatively low level throughout the entire processing cycle.

The data also suggest that monitoring soluble signals might be used to determine whether there had been any bacterial buildup during any stage of the reclamation process.

Reactor Cell Studies*

The effect on signal response of modifying the design of the reactor and its reflecting surface was investigated. The results of these studies are described briefly below.

1) Reflector Modification

Up to this stage of development, the only reflecting surfaces for the light generated in the reactor cell were those provided by the aluminum walls of the reactor cell mount and housing. The effect of backing the reactor assembly (Figure 16) with a dome-shaped aluminum reflector to provide for a more efficient collimation of the generated light is shown in Table 16. The data indicate an average improvement in signal strength of approximately 40%.

Additional runs were performed with the modified system; with 1 volt regarded as a significant signal, the results shown in Table 17 indicate detection thresholds of approximately 75 and 150 cells/ml (100 ml sample for E. coli and S. marcescens.

Higher sensitivities might be achieved by use of larger samples (i.e., 500 vs 100 ml) or coupled optics to increase the light gathering efficiency of the system, or as shown below, by improving reactor cell geometry.

2) Reactor Cell Geometry

A brief examination was made of the effect of masking out the waste leg of the reactor cell (shown by hatched area in Figure 17) to determine if the major portion of the light was generated in that section of

*The reflector modification described in this study was made subsequent to the analysis of the reclaimed water samples from McDonnell-Douglas.

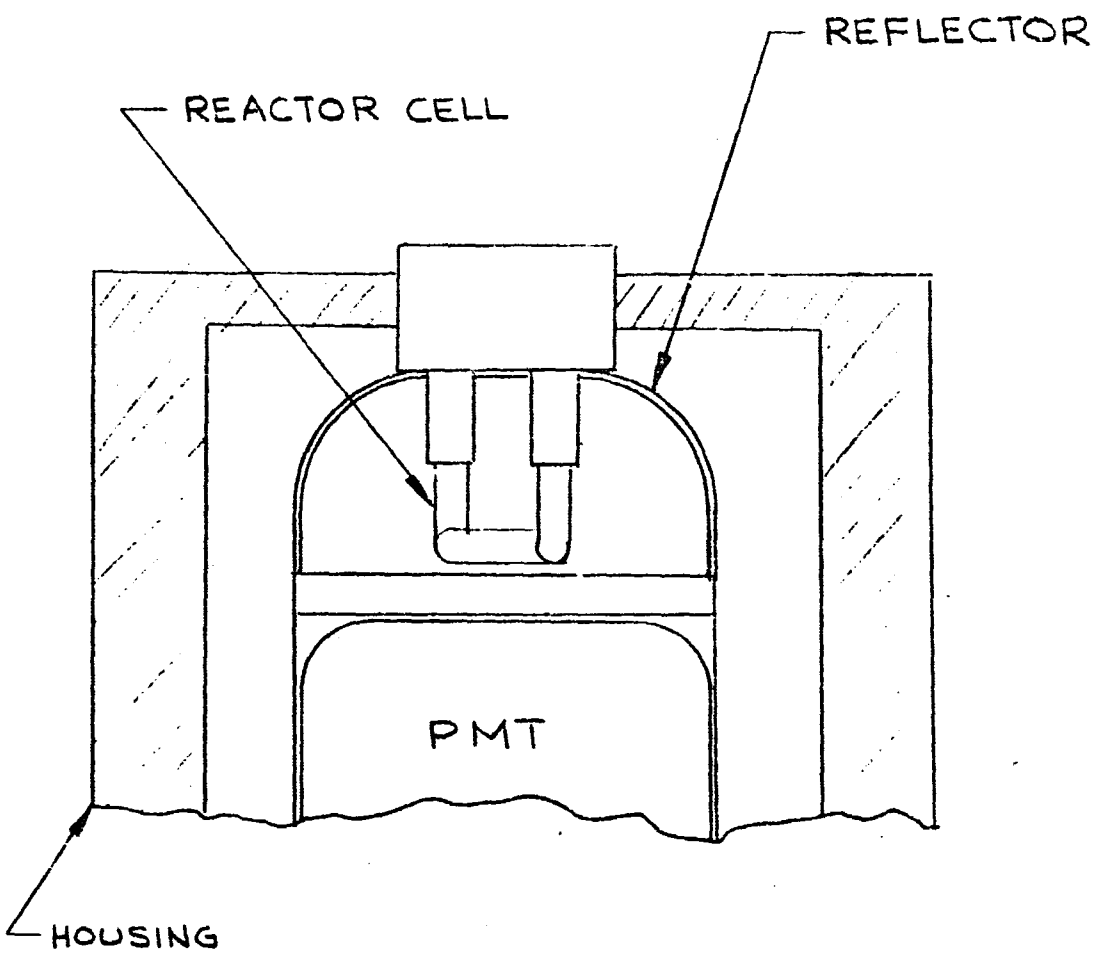

FIG. 16

REFLECTOR IN PMT/REACTOR HOUSING

Table 16

EFFECT OF ADDED REFLECTOR ON SIGNAL STRENGTH

		Net Signal* (volts)
With Added Reflector		14.5
		16.0
		19.0
		19.0
		15.0
		19.0
		13.5
	av =	16.6
Without Added Reflector		10.0
		12.0
		10.5
		10.5
		12.5
		14.5
		13.0
	av =	11.9

*A 10 ml water sample containing 1×10^4 E. coli/ml was processed according to protocol in Table 1.

Table 17

DETECTION THRESHOLD FOR TOTAL COUNT OF E. coli AND S. marcescens

(With Reflector)

Organism	Total Cells/ml*	S-N Net Signal, volts
E. coli	50	0.5
	75	1.0
	100	2.5
		2.5
		3.0
		1.0
		X = 2.1
S. marcescens	150	1.5
	200	5.2

*100 ml sample processed according to protocol shown in Table 1.

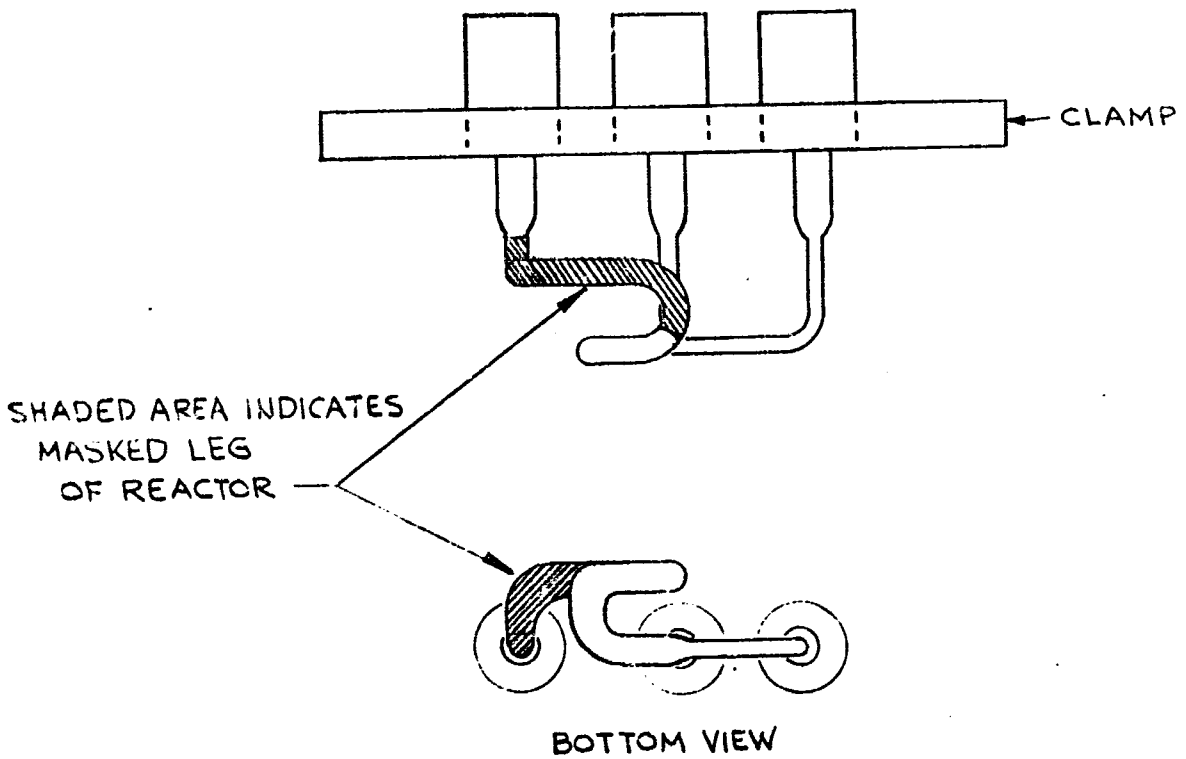

FIG. 17

MASKING OF REACTOR LEG

the reactor closest to the PMT cathode. The results obtained with a single bacterial concentration (1×10^4 E. coli/ml, 10 ml sample) and the protocol in Table 1, were as follows:

	Net Signal, volts
Reactor with mask	4.5 5.5
Reactor without mask	18.5

The data indicate that, the waste leg of the reactor, which is approximately 3/8" farther back from the PMT than that portion of the reactor closest to the mixing zone, contributes about 73% of the total light. The actual residence time in that portion of the reactor closest to the PMT is about 11 seconds,* which should be adequate considering the rapidity of the reaction. It would appear from these data, however, that a more optimal reactor design would be one with a larger volume or one in which the waste leg of the reactor were moved closer to the PMT. It is apparent that further optimization of the cell configuration could lead to a significant improvement in sensitivity of the system.

Summary & Conclusions of Protocol Development Studies For Total Cell Count

The sensitivity obtained for each stage of protocol development can be evaluated by comparing the signal obtained at a single bacterial level, thus

Stage	Protocol	Av. Net Signal (volts) (1×10^4 E. coli/ml-10 ml
1	Single Cycle Protocol (Table 3) (2M urea backwash)	5.5

*The liquid holdup volumes of the waste leg and main portion of the reactor are 0.42 and 0.30 cc, respectively. Residence times (at 1.7 ml/min total flow) are 15 and 11 seconds respectively.

Stage	Protocol	Av. Net Signal (volts) (1×10^4 E. coli/ml-10 ml
2	Double Cycle Protocol (Table (2M urea - first backwash)	
	13 mm first filter (2 ml/min)	6.4
	25 mm first filter (2 ml/min)	11.3
	25 mm first filter (4 ml/min)	11.4
3	Double Cycle Protocol (Table 1) (Dextrose broth - first backwash)	
	Unmodified reflector (25 mm first filter)	12.0
	Modified reflector (25 mm first filter)	17.5

The increase in signal from 5.5 to 11.4 volts at a fixed bacterial challenge is due mainly to an increase in bacterial recovery efficiency brought about by the use of a larger diameter first filter(i.e., 25 mm vs 13 mm). Since actual bacterial recoveries measured with the 13 mm filter ranged from 58 to 66%, it is apparent that with the 25 mm filter, the overall recoveries must be virtually quantitative ($\sim$100%). The further increase from 12 to 17.5 volts in the net signal is due principally to the more efficient reflection of the generated light. Aside from the use of larger samples, further increases in sensitivity might be achieved by even more optimum coupling of the reactor to the PMT as well as a more favorable reactor cell geometry.

In summary, the best sensitivity was achieved with the Stage 3 protocol. Detection thresholds obtained for 100 ml samples using this protocol were found to be 75 and 150 cells/ml for E. coli and S. marcescens, respectively.

In a study of the effect of water immersion on signal strength, a suspension of E. coli in distilled water exhibited a drop of only 25% in signal strength after 24 hrs at ambient temperature (i.e., from 16 to 12 volts for 1.4×10^4 cells/ml - 10 ml sample).

3.2.2 INCUBATED CYCLE (For monitoring viable cells)

In determination of water sterility, the signals of an incubated and unincubated sample, both subjected to the same processing sequence, are compared. A higher signal for the incubated sample indicates the presence of viable organisms.

In establishing the optimum procedure for processing the incubated sample, a number of ancillary experiments, concerned with selection of the proper nutrient and conditions of incubation were performed. The protocol which finally evolved provided maximum sensitivity not only for the viable (20-40 cells/ml, 100 ml sample) but the non-viable cycle as well. The studies which led to the final selection shown in Table 2 are described briefly below.

3.2.2.1 EVALUATION OF GROWTH MEDIA (For Use in Incubation)

Several general purpose media suitable for growing fastidious and non-fastidious pathogens were examined for the possible presence of porphyrin components which might produce background signals in the chemiluminescent reaction. For the evaluation, a chemiluminescent detection device developed for the U.S. Army was utilized. This instrument contains a filter-concentrator which automatically filters and concentrates the organisms in a liquid stream using one port of an 8-port disc. The disc is indexed periodically to a new station where the organisms are washed and then backwashed and reacted with luminol reagent in a glass cell mounted on the face of a photomultiplier tube.

The net chemiluminescence signals obtained when different media were fed into the collect port of this device are shown in Table 18. The net signals shown are those generated by traces of soluble porphyrins which may have remained in the port cavity after the water wash cycle.

The lowest signals were obtained with Tryptose, Tryptose Phosphate and Trypticase Soy, followed by Dextrose and Columbia broths.

The relative growth of E. coli and S. marcescens in those media which had exhibited minimal background interference was evaluated by innoculating with a fixed bacterial challenge and incubating

Table 18

NET CHEMILUMINESCENCE SIGNAL
OF
NUTRIENT BROTHS IN THE CDS*

	Net Signal (Volts)
Difco Dextrose Broth	1.0
Difco Tryptose Broth	0.5
Difco Tryptose Phosphate Broth	0.5
BBL Trypticase Soy Broth	0.5
Difco Todd-Hewitt Broth	3.0
BBL Columbia Broth	1.0

*CDS - Automated Chemiluminescent Detection System built for the U.S. Army; 0.2μ Acropor filters, 1/2-minute cycles of collect, wash and backwash (sterile distilled water).

under controlled conditions*. Bacterial growth was monitored by absorbance measurements at 640 mμ using a Bausch and Lomb 340 Nephelometer. A summary of the relative growth**, shown below, indicates that Dextrose broth was superior to all others tested.

	Relative Concentrations**			
E. Coli	Dextrose Broth	Tryptose Broth	Trypticase Soy Broth	Tryptose Phosphate Broth
Exp. No. 1	4.6	2.3	-	1
Exp. No. 2	3	-	1	-
S. Marcescens				
Exp. No. 3	2	-	1	-

3.2.2.2 EFFECT OF OXYGEN ON BACTERIAL GROWTH

The effect of growing E. coli over a limited period in Dextrose broth containing only dissolved oxygen was investigated as an aid in establishing the design parameters of the incubator. A simplified incubator design (without hydrophobic filters) could be utilized if it could be shown that the dissolved oxygen normally contained in nutrient broth is sufficient to sustain growth over the interval in question. In the following experiment, test tubes (1/2 x 4") were filled 2/3 full with Dextrose broth containing E. coli (2×10^5/ml) and incubated at 31°C for periods up to 5 hours. One set of duplicate tubes contained a (1/2") layer of sterile mineral oil to exclude air, the other set a headspace of air and a sterile cotton plug. Bacterial growth was monitored by absorbance measurements as before. The results summarized in Table 19 indicate that after 5 hours incubation, the bacterial levels were the same in both sets of tubes. Apparently, a supplemental oxygen supply is not needed for growing the bacteria (at an initial inoculum level of 2×10^5/ml) over a 5-hour interval.

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*Incubation with 18 hr culture at 31-32°C for 4-5 hours, 100 ml nutrient in 250 ml flasks with sterile styrofoam stoppers.

**Relative bacterial concentrations at end of incubation period.

Table 19

GROWTH OF E. coli UNDER LIMITED OXYGEN SUPPLY AT 31°C*

Incubation Time	With Sterile Mineral Oil	Without Sterile Mineral Oil
0 Hours	2×10^5/ml	2×10^5/ml
2 Hours	$< 10^6$	$< 10^6$
4 Hours	6×10^6	9×10^6
5 Hours	2.5×10^7	2.5×10^7

*Initial inoculum contained 2×10^5 E. coli/ml in Dextrose broth; bacterial numbers are per ml as determined from absorbance measurements.

To determine whether there was any difference in the ability of these organisms to initiate chemiluminescence, the 5-hour cultures were diluted to 1.5×10^4/ml and fed into the automated Army chemiluminescent device described earlier*. The net chemiluminescence signal obtained for each of the sample preparations was identical; namely, 2 volts, indicating no reduction in the bacterial porphyrin content.

3.2.2.3 BACTERICIDE SCREENING

Several bactericides were evaluated for their effect on the chemiluminescence reaction and for their effectiveness in checking bacterial growth. Bacterial buildup in the chemiluminescence system is minimized by flushing with bactericide between incubation cycles. Other features considered desirable for a bactericide in the present application include non-toxicity, good shelf life, compatibility with materials of construction and one easily flushed from the system.

In an attempt to simulate the manner in which the bactericide would eventually be used, the bactericide (diluted according to manufacturer recommendations) was passed through a filter**, the filter washed with water (1 ml/min for 1 min) and then backwashed with 8M urea (1 ml/min for 1 min). The net signal obtained and the ease with which the signal returned to a baseline value after the bactericide flow was cutoff, are summarized by the data below.

Bactericide	Active Ingredients	Dilution	Net Signal	Return to Normal Reagent Background
Consan-20 (Consan Pacific)	mixture of alkyl benzyl ammonium chlorides	1:512	-1 volt	rapid
Chlorophenyl (Bard-Parker)	hexachlorophene	full strength	-1 volt	slow (1 hr to clean)
Amphyl (Lehn & Fink)	mixture of a ricinoleate, phenols and alcohol	1:200	+1 volt	rapid
Celldex (Davis Labs)	organic perioxide	full strength	+3 volts	rapid

*At 1/2 minute cycles, sample was collected (liquid flow 1 ml/minute at 1.5×10^2/ml), washed with water, and then backwashed with 8M urea into the reactor.

**U.S. Army Chemiluminescence Device with indexing filter-concentrator was used for this screening.

Of the four tested, Consan-20 appeared to contain most of the desired features. The killing effectiveness of this agent was evaluated against E. coli with the following results:

	Plate Count
Viability of E. coli suspension (before exposure) =	4.4×10^4/ml
Viability after 5 min exposure to Consan-20 (1:512 dil) =	3/ml

Although used initially, Consan-20 was later replaced by 8M urea which was more effective in removing residual protein and more easily flushed out of the system.

3.2.2.4 TESTS FOR VIABILITY (STAGE 2 PROTOCOL)

Initial tests for water sterility were performed using the Stage 2 protocol shown in Table 4. In this sequence 2M urea served as the backwash fluid for both (13 mm dia.) filters. Incubation was at 32°C and the incubation mixture consisted of backwashed organisms (in 2M urea) and Dextrose broth in a 1:1 ratio. With this protocol, it was found that the increase in signal observed on incubation inside the water monitor was only a fraction (10-15%) of that expected on the basis of a direct microscopic count of an aliquot incubated in capped test tubes outside the system. This is illustrated by the data of two runs summarized in Table 20, one a 10 ml and the other a 100 ml sample. Actual recorder traces for incubated and unincubated samples of 10 ml are shown n Figures 18 and 19.

Referring to the data for the 10 ml sample, a 6-fold increase in signal occurred after a 5-hour incubation. After 4 hours incubation, the bacterial count on an aliquot incubated outside the system had increased from 5×10^3/ml to 1.4×10^5/ml, a factor of approximately 30. Although not actually determined, the estimated multiplication factor after 5 hours incubation should have been at least 60X. Thus the actual signal in the water monitor increased only about 10% of that observed outside the system.

Similarly for Run No. 2 (Table 20). Since growth in the presence of 2M urea often produces abnormal morphologies, two sets of values are given for the bacterial counts at 6 and 7 hours, one based on counting a chain as a single cell and the other (in parenthesis) on estimating the number of cells in an individual chain. Based on an extrapolated value for the lower counts of about 2×10^5/ml for an 8-hour incubation outside the system, this represents about a 20-fold increase in bacterial concentration. Incubation inside the water monitor produced only about a 3-fold increase in signal (or 15% of theoretical).

This apparent lack of agreement between growth inside and outside the system was subsequently shown to be due to two factors, namely:

Table 20

VIABILITY DATA
(Inside vs. Outside Incubation)

RUN NO. 1

Inside Incubation

Sample Size	Total E. coli/ml**	Viable*** Plate Count Cells/ml	Net Chemiluminescence Signal++, volts: Unincubated Sample	Net Chemiluminescence Signal++, volts: Incubated Sample (5 hrs 32°C)
10 ml*	5×10^3	1.9×10^3	2.5	15

Outside Incubation (in Capped Test Tubes)

Direct Microscopic Counts*, cells/ml: Initial	Direct Microscopic Counts*, cells/ml: After 4 hrs at 32°C
5×10^3	1.4×10^5

RUN NO. 2+

Inside Incubation

Sample Size	Total E. coli/ml	Viable Plate Count Cells/ml	Net Chemiluminescence Signal++, volts: Unincubated Sample	Net Chemiluminescence Signal++, volts: Incubated Sample (8 hrs 32°C)
100 ml*	100	36	3	8

Outside Incubation (in Capped Test Tubes)

Direct Microscopic Counts*, Cells/ml: Initial	5 hrs Incubation	6 hrs Incubation	7 hrs Incubation
1.0×10^4	1.5×10^4	$\sim 6.6 \times 10^4$ ($\sim 2 \times 10^5$)	$\sim 9.6 \times 10^4$ ($\sim 4 \times 10^5$)

Morphology			
Small rounded rods	Cells variable; few original size, most several microns in length	Cells 5-15 μ long, few original size	Cells in long chains (10-20 μ)

*Concentrated at 2 ml/min.

**Total - by direct microscopic count.

***Viable - by standard pour plate on TGE agar.

+E. coli had been in contact with distilled water for 3-1/2 hrs at ambient before assay.

++Signal above reagent baseline (uncorrected for water blank)

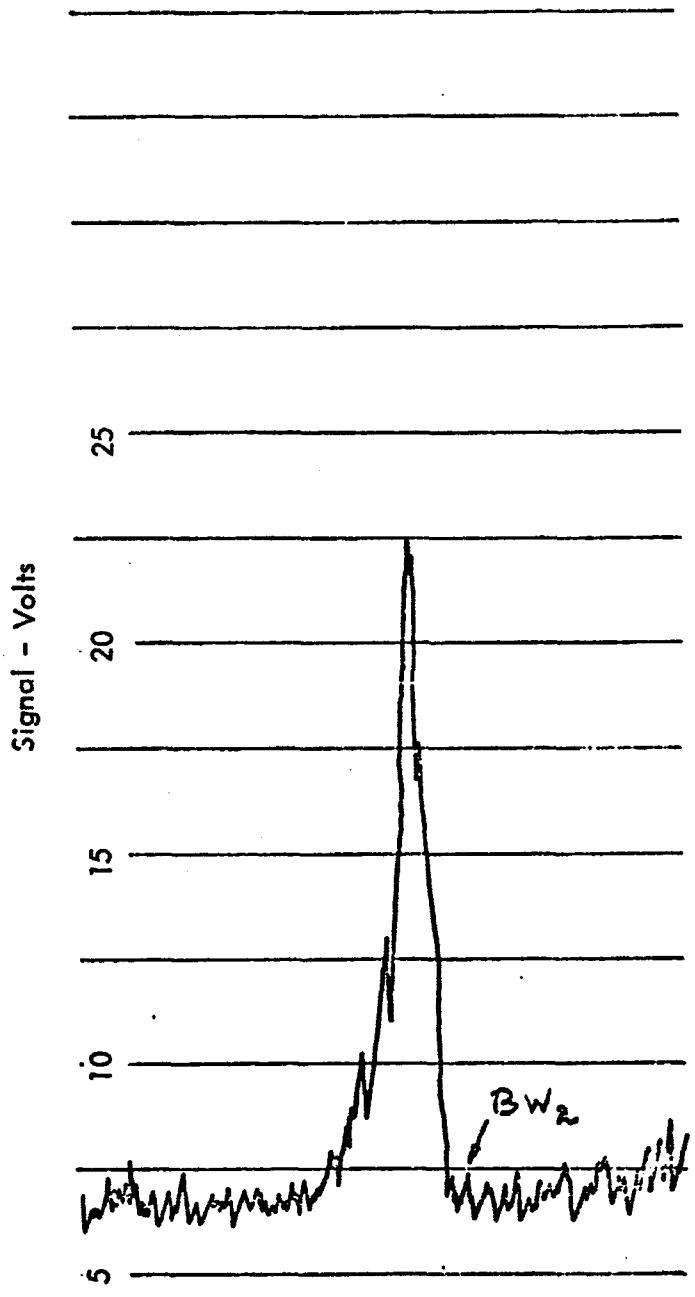

Figure 18

E. coli RESPONSE - INCUBATED 5 HOURS AT 32°C
(5×10^3 total cells/ml -- 1.9×10^3 viable cells/ml, 10 ml sample)

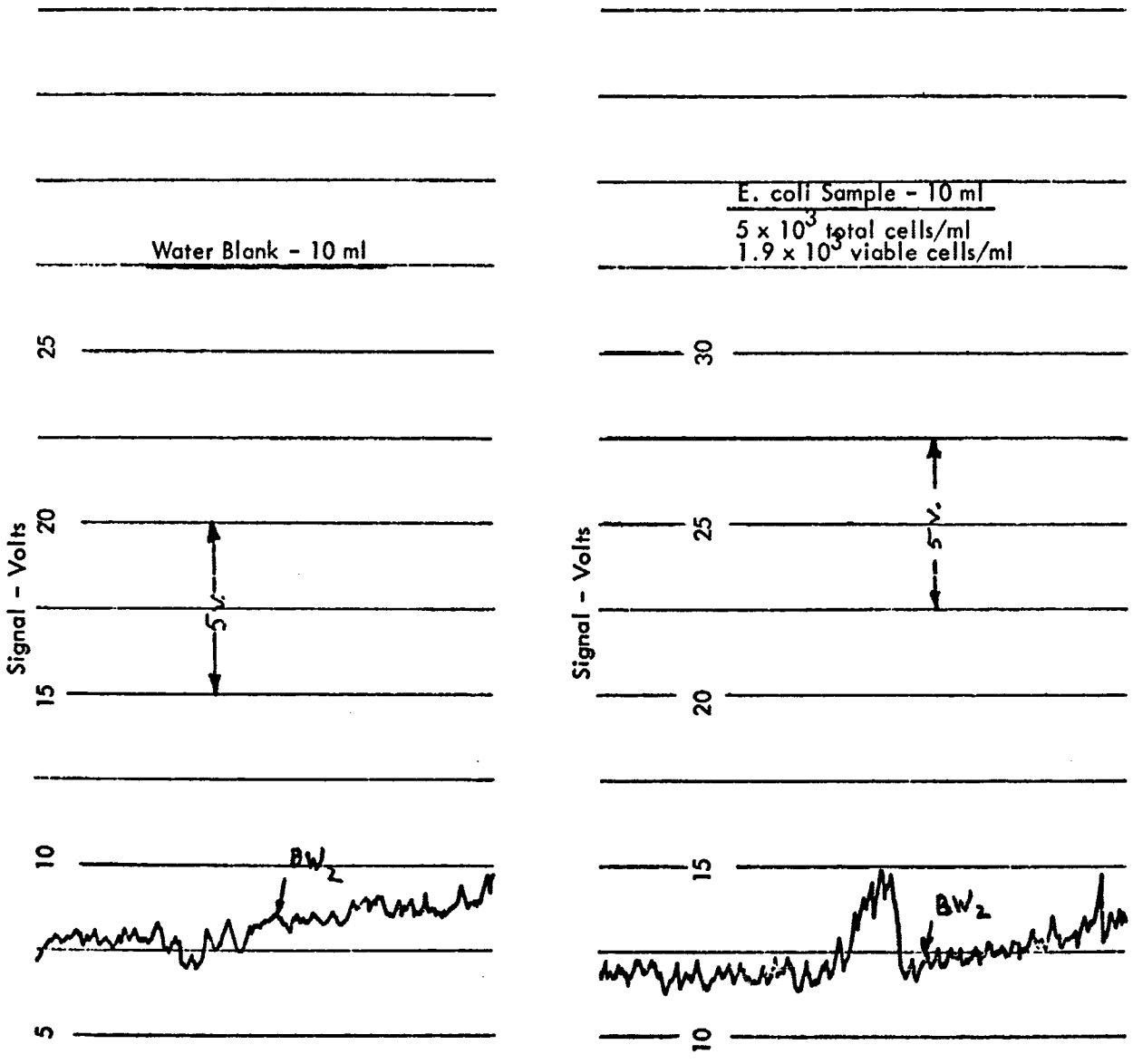

Figure 19

UNINCUBATED E. coli SAMPLE AND WATER BLANK RESPONSES

1) Settling of the bacterial suspension in the vertical incubator during incubation so that the bulk of the organisms was not in the heating zone.

2) Impairment of bacterial cell division by the more anaerobic conditions of internally incubated samples.

Both of these problems were eliminated by using a horizontal incubator with Dextrose broth as a first backwash.

Settling or migration of the bacteria out of the incubation zone during the 5-hour incubation period is indicated by the data in Table 21; a vertical (spiral) coil was used for incubating internally. The fact that the concentration of the internally incubated sample was only a fraction of the expected minimum even after a 5-hour incubation can best be attributed to settling. A repeat run made with the incubator in the horizontal position (Table 22) shows much better agreement between internally and externally incubated water samples. Thus, the sample incubated outside the system exhibited a multiplication factor of 4, whereas the internally incubated sample, a multiplication factor of approximately 4 or 8, depending on whether the recovery was 50 or 100%. It is to be noted that the bacterial counts shown in Table 22 were derived from an estimation of the number of organisms per chain (some of the cells exhibited abnormal growth - i.e., increasing in length without undergoing cell division) rather than counting each chain as a single cell. If the long filaments were counted as single cells, the increase in the number of cells would turn out to be small or negligible. It was generally observed that internally incubated samples contained more of these abnormal shaped cells. The reason for this becomes apparent from examination of the data shown in Tables 23 and 24. The results show that the combined effect of urea and anaerobic conditions favors abnormal growth. This effect is even noted at urea dilutions of 1:3 and becomes less apparent at urea dilutions of 1:9.

Also evident is the fact that an auxiliary air supply is not required for growth in Dextrose broth under the conditions employed. Whereas growth of E. coli in nutrient broth is more prolific at 37°C than 32°C, this effect of temperature is nullified in the presence of urea.

The effect on bacterial growth of extended storage to distilled water may also be noted. Referring to an experiment in which E. coli had been immersed in distilled water for 2 hours and 7 days respectively,

Table 21

SETTLING OF BACTERIA DURING INCUBATION

Procedure:

E. coli bacteria were incubated in an external incubator at 32°C for [illegible] and the total (direct microscopic) count compared to the total count of E. coli incubated internally for a like period. Both incubations were in 1:1 2M urea and Dextrose broth. Internal incubator - vertical spiral. External incubator - capped test tubes.

Internal Incubator:

Sample size 10 ml of 4×10^3/ml (4×10^4 total)

Minimum expected concentration in incubator* 1×10^4/ml (start of incubation)

Concentration found after 5 hours incubation - 2.8×10^3/ml** (many long winding chains)

External Incubator:

Initial concentration - 2×10^4/ml

Concentration after 5 hours incubation - 8×10^4/ml** (many normal shaped cells, few long chains, few large round cells)

*Assuming 50% recovery on backwashing; backwashed suspension is diluted 1:1 with 2M urea before entering the incubator.

**Chains counted as a single cell.

Table 22

COMPARISON OF INTERNAL AND EXTERNAL INCUBATORS (HORIZONTAL INCUBATOR)

Test Procedure:

E. coli were incubated in an external incubator at 32°C for 6 hours and the total count compared to a sample incubated internally. Both were incubated in 1:1 2M urea and Dextrose broth. Internal incubator - horizontal.

Internal Incubator:

Sample size 10 ml of 8×10^3/ml (8×10^4 total)

Minimum expected concentration in incubator* 2×10^4/ml (start of incubation)

Concentration after t hours incubation - 1.5×10^5/ml** (many long chains, few round, few normal)

External Incubator:

Initial concentration - 4×10^4/ml

Concentration after 6 hours incubation - 1.6×10^5/ml** (few long chains, few round, many normal)

*Assuming 50% recovery on backwashing; backwashed suspension is diluted 1:1 with 2M urea before entering the incubator.

**Cells counted by estimating number of organisms/chain.

Table 23

GROWTH STUDIES OF E. coli

Test Procedure:

Stock E. coli (at $\sim 10^{10}$/ml) in distilled water at refrigerator temperature ($\sim 5^{\circ}C$) 1 week was used for preparing all of the following solutions containing 1.4×10^{4} cells/ml. Incubation in capped test tubes for 4 hours at $37^{\circ}C$. Direct microscopic counts of incubated samples follow:

Dextrose Broth (DB)	DB with Sterile Oil Layer	1:1 DB + 2M Urea	1:1 DB + 2M Urea + Oil Layer	3:1 DB + 2M Urea	3:1 DB + 2M Urea + Oil Layer
9.3×10^{5}/ml	2.7×10^{6}/ml	4.6×10^{4}/ml	1.4×10^{4}/ml	4.5×10^{4}/ml	1.3×10^{4}/ml
Normal Shape	Normal Shape	Normal Shape, Small in size	Few Chains	Normal Shape, Small in size	Few Chains
66*	193	3.3	0	3.2	0

*Multiplication factor over initial concentration.

Table 24

GROWTH STUDIES OF E. coli

Test Procedure:

Stock E. coli ($\sim 10^{10}$/ml) in distilled water at refrigerator temperature ($\sim 5^{\circ}C$) 3 days used for preparing 1.2×10^{4}/ml dilutions in the following. Incubation in capped test tubes for 4 hours at indicated temperatures. Direct microscopic counts for incubated samples follow:

Dextrose (DB) 32°C	DB 37°C	1:1 DB + 2M Urea 32°C	1:1 DB + 2M Urea 37°C	9:1 DB + 2M Urea 32°C
8.4×10^{4}/ml	3.1×10^{6}/ml	4.3×10^{4}/ml	4.6×10^{4}/ml	6.5×10^{4}/ml
Normal Shape	Normal Shape	Small in Size, Normal Shape	Small in Size, Normal Shape	Normal Shape and Size
(7*)	(260)	(3.6)	(3.8)	(5.4)

*Multiplication factor over initial concentration

the following was observed:

Exposure Time of E. coli to Distilled Water	Initial	Direct Microscopic Count, Cells/ml 2 hrs Incubation +	4 hrs Incubation +	Morphology
7 days (at ~ 5°C)	1.5×10^{5}*	1.1×10^{5}	1.6×10^{5}	Cells begin to approach normal size after 4 hrs incubation
2 hours (at ambient)	2.8×10^{5}**	6.1×10^{5}	1×10^{7}	Cells reached normal size in less than 2 hrs incubation

The above data indicate that although significant viability (i.e., 28%) is still retained after one week immersion in distilled water*, these viable organisms require at least 4 hours or more to fully recover from their injury caused by immersion in distilled water. During this interval, microscopic observation indicated a change in morphology from small rods almost coccoid in form, to larger elongated rods typical of E. coli in the log phase of growth. In marked contrast, those E. coli which were suspended for only 2 hours in distilled water prior to testing exhibited a normal morphology and significant replication in less than two hours. The rate of recovery (i.e., lag period) of stressed organisms would depend not only on the period of immersion in water but on the make-up of the growth medium as well. Recoveries would be expected to occur more rapidly in dextrose broth than in mixtures of dextrose broth and urea. Thus, much shorter incubation periods were required to produce the same level of growth when urea was eliminated from the incubation mixture.

Higher sensitivity in the viable cycle was also achieved on raising the temperature of incubation. The data which supports this change are shown in Table 25. The data indicate that the best common

*28% viable by 24 hr growth on TGE (pour plate).

**73% viable by 24 hr growth on TGE (pour plate).

+Incubation in a 1:1 mixture of 2M urea and Dextrose broth.

temperature for growing both E. coli and S. marcescens appears to fall between 35 and 37°C. The latter temperature was selected for all subsequent incubations.

3.2.2.5 TESTS FOR VIABILITY (STAGE 3 PROTOCOL)

Some of the principles derived from the preceding studies were utilized in formulating the Stage 3 protocol shown in Table 2. The results of a number of experiments performed for E. coli and S. marcescens are shown in Tables 26 and 27. Regarding 1 volt as a significant signal, the data indicate that 20 to 30 viable cells of E. coli or S. marcescens/ml (100 ml sample) can be detected in a 2 hour incubation at 37°C. This is decidely better than the 8-hour incubation (at 32°C) required for detection of 36 viable E. coli/ml (100 ml sample) with Stage 2 protocol in Table 4.

One additional operational change that was introduced was the use of multiple-incubators. In order to increase the amount of data attainable within a single working day, a procedure was developed in which several incubators were charged successively with a bacterial challenge and removed for incubation outside the system. Following incubation, the incubators were returned to the system and then processed for signal output in the normal manner. A detailed operational sequence for the viable and non-viable cycle is given in Appendix A.

Table 25

GROWTH STUDIES OF *E. coli* AND *S. marcescens*

Conditions: Fresh (16 hr) bacterial cultures used. Growth was in dextrose broth. Bacterial numbers determined by direct microscopic count of stained organisms.

E. coli		*S. marcescens*	
Initial Conc:	1.5×10^4/ml	Initial Conc:	1×10^4/ml

Multiplication Factors

q	2 Hr.	4 Hr.	2 Hr.	4 Hr.
32°C	3.3	100	9.2	690
35°C	4.7	247	7.6	660
37°C	5.0	430	3.8	430

Table 26

DETECTION THRESHOLD FOR VIABLE E. coli

(Stage 3 Protocol)

Exp.	E. coli (100 ml sample)	Reflector Modification	Net Signal (volts)* Unincubated	Incubated	Incubation Time
1A**	100/ml (total) 69/ml (viable)	No	1.5	20	4 hrs
1B	100/ml (total) 69/ml (viable)	No	1.5	19	4 hrs
2A	100/ml (total) 60/ml (viable)	No	0.8	5.8	2 hrs
2B	50/ml (total) 30/ml (viable)	No	0.5	1.8	2 hrs
3	100/ml (total) 22/ml (viable)	Yes	2	7.5	2 hrs
4A	100/ml (total) 76/ml (viable)	Yes	1	5	2 hrs
4B	50/ml (total) 32/ml (viable)	Yes	0.5	1.5	2 hrs
5A***	100/ml (total) 58/ml (viable)	Yes	0.8	1.3	2 hrs
5B***	50/ml (total) 28/ml (viable)	Yes	0	1.0	2 hrs

*Corrected for water blank.

**A & B samples run consecutively using multiple incubators.

***Samples 5A and 5B were aliquots of 4A and 4B which had remained 24 hrs at ambient temperature (E. coli in distilled water).

Table 27

DETECTION THRESHOLD FOR VIABLE
S. marcescens

(Stage 3 Protocol)

Exp.	S. marcescens (100 ml)	Reflector Modification	Net Signal (volts)* Unincubated	Net Signal (volts)* Incubated	Incubation Time
6A**	100/ml (total) 20/ml (viable)	No	0	2	2 hrs
6B	100/ml (total) 20/ml (viable)	No	0.5	2.3	2 hrs
7	200/ml (total) 33/ml (viable)	Yes	5.8	13.5	2 hrs

*Corrected for water blank

**A and B samples run consecutively using multiple incubators.

3.2.3 REAGENT SHELF LIFE STUDIES

Studies to define the shelf life of reagents (i.e., luminol, H_2O_2, nutrient, 2M and 8M urea) used with the present system were carried out with the following results.

3.2.3.1 LUMINOL-H_2O_2

Premix Reagent

The Premix reagent which is used in the water monitor is formed by mixing stock luminol and H_2O_2 solution in a mixing coil with a 1/2 hour delay before being permitted to react with the bacterial suspension. The 1/2 hour delay is required in order to achieve maximum sensitivity with this reagent. Supporting evidence for this is presented in Table 28. The data were obtained by reacting Premix prepared from a single batch of stock luminol with a single suspension of E. coli. For convenience, measurements of net luminescence were made using a commercial chemiluminescence unit developed at Aerojet.

Aged Luminol and H_2O_2

The comparative signals* generated against a single concentration of E. coli (5×10^3 cells/ml - 16 ml) by aged and unaged luminol and H_2O_2 were determined. In this experiment luminol and H_2O_2 (at usable concentration) aged for 5 weeks at ambient temperature (in polypropylene containers with snap-on lids) were compared to fresh solutions prepared that day. The net signal obtained by each were essentially the same (fresh luminol-H_2O_2 gave 18 volts vs 17 volts for the aged solutions).

The long term stability of the luminol solution itself was evaluated by utilizing luminol which had been aged for periods ranging from 1 day to 4 months (see Table 29). In this evaluation all of the luminol samples were mixed with fresh H_2O_2 and the resulting mixtures (Premix) tested 2-1/2 hrs after mixing against a single batch of E. coli (normalized to a single concentration). The data from this series summarized in Table 29 indicate adequate reproducibility in sensitivity for luminols stored up to 4 months at ambient.

*Using Stage 3 protocol.

Table 28

EFFECT OF AGING OF PREMIX REAGENT ON SENSITIVITY

Time after Mixing*	Net Chemiluminescence Signal for E. coli Suspension**, volts
5-25 min	7.09
30-50 min	8.87
60-80 min	8.74
120-140 min	8.01
	$\bar{X}$ = 8.54 S = ±0.38

*Represents time interval over which measurements were made.

**Average of 10 readings made over indicated interval in a commercial Aerojet Chemiluminescence Unit.

Table 29

EFFECT OF AGING STOCK LUMINOL ON SENSITIVITY OF PREMIX REAGENT

Age Luminol	Net Bacterial Signal (S-N, volts)+
1 day (PP*)	7.82
3 weeks (PP)	8.45
3-1/2 weeks (PP)	7.99
2 months (PP)	8.78
4 months (PE*)	8.62
4 months (PP)	9.44
	$\bar{X}$ = 8.48 S = ±0.62

Coefficient of Variation $\frac{S}{\bar{X}}$ = ±7.3%

*PP - stored in translucent polypropylene

*PE - stored in brown polyethylene

+Measurements made in a commercial Aerojet Chemiluminescence Unit.

Aged 2M Urea

Since 2M urea is used as a wash and backwash for the 2nd filter, the effect of aging on recovery of backwashed organisms was evaluated against a single concentration of organisms using the Stage 3 protocol. A 2M urea solution which had been aged for 5 weeks at ambient temperature in a glass bottle (plastic screw cap), although water clear contained trace amounts of particulate. The origin of the latter was probably the plastic cap. The solution was filtered through a 0.22 μ Millipore and the clear filtrate produced a net signal identical to that for fresh 2M urea (i.e., 18 volts for a challenge of 5×10^3 E. coli/ml - 16 ml sample.)

Aged 8M Urea

Urea (8M) is used principally as a bactericide and to remove residual protein from the system (particularly the 2nd filter). Urea (8M) aged 5 weeks at ambient temperature (in a glass bottle with plastic cap), although remaining essentially clear, showed some evidence of particulate formation (probably from the plastic cap). The 8M urea solution was filtered and its performance compared with an unaged 8M urea solution with respect to its ability to prevent cross-contamination between runs. The results indicated no significant difference in performance between aged and unaged urea solutions.

Aged Nutrient (Dextrose Broth)

The functions of the Dextrose broth in the Stage 3 protocol are 1) to serve as a wash and backwash fluid on the first filter and 2) to serve as a nutrient in the incubation step of the viable cycle.

The first property was evaluated by comparing the net signal obtained for aged (5 weeks at ambient temperature*) and unaged nutrient against a single bacterial challenge (100 E. coli/ml - 100 ml sample). The net signal obtained in both cases was 1.5 volts indicating no affect on aging.

The second property was evaluated by comparing the rate of growth of E. coli in aged and unaged nutrient. Test tubes containing aged and unaged nutrient were innoculated with E. coli (final concentration 2×10^3/ml) and incubated at 37°C for 4 hours (Prior to this, the sterility of the aged nutrient was checked by aging overnight at 37°C and checking for bacterial growth. No growth was evident as indicated by visual inspection and optical density measurement at 525 mμ).

A direct microscopic count made at the end of the incubation period indicated the following:

Initial Concentration	2×10^3 cells/ml
Final Concentration	
Fresh Broth	2×10^5 cells/ml
Aged Broth	5×10^4 cells/ml

The data indicate that the aging process had caused some deterioration of the nutrient properties of the Dextrose broth. This was also evidenced by a difference in color of the two broths, with the aged solution having turned a lighter shade of amber (although still clear). Storing in the dark at perhaps 5-10°C may be useful in prolonging the shelf life of the nutrient broth.

*Nutrient aged in the container in which it is normally used, namely a sterile AGI (All Glass Impinger) with a cotton plug.

Section 4

CONCLUSIONS AND RECOMMENDATIONS

The maximum sensitivity for total and viable cells was achieved with the Stage 3 protocol outlined in Tables 1 and 2. The operational procedure entails processing the water sample through two cycles of concentration, washing and backwashing with an incubation step (for viables) in between. Dextrose broth was used as the wash and backwash fluid in the first cycle, 2M urea in the second.

The final sensitivities achieved for total and viable cells with a 100 ml sample are shown below. Although not specifically indicated as a program objective, desired sensitivities are included below for comparison:

	Total (Viable + Non-Viable)		Viable	
	Sens.	Processing Time	Sens.	Processing Time
Desired Goals	10-100 cells/ml	$\leq$ 1 Hr.	10-100 cells/ml	$\leq$ 3 Hrs.
Final Sensitivity	75 E. coli/ml 150 S. marcescens/ml	51 Min.	20-30 cells/ml E. coli & S. marcescens	< 3 Hrs.

The data indicate that the desired sensitivities and processing time were achieved for E. coli on total and viable and for S. marcescens on viable only. Aside from increasing sample size, the studies conducted thus far indicate that further improvement in sensitivity might be achieved by increasing the light gathering efficiency of the generated light. This might be accomplished by use of better reflectors and a modified reactor design which permits more optimum coupling to the photomultiplier tube.

An estimate of the sensitivity for total cells which might be achieved under favorable conditions can be made from consideration of the following:

Concentration of a 200 ml sample (at 10 cells/ml) should provide a total of 2000 organisms. Based on data derived with other similar chemiluminescence systems, the light generated in chemiluminescence reaction is typically 0.015 picowatt/organism* so that 30 picowatts of detectable light would be released. With suitably coupled optics, it should be possible to transmit at least 30% of the spherically emitted light to the photocathode of the photomultiplier, or 9 picowatt.

*For S. marcescens

The peak radiant sensitivity of a new EMI 9635B photomultiplier is typically 75 ma/watt (4300A°), which is equivalent to 70 ma/watt for the luminol spectrums. Hence, 0.63 picoamp will be generated at the photocathode.

For typically 1500 volts (1200-2000 volt range) a gain of 2.5×10^7 is available, which would amplify the current to 16 microamp. Selection of a 100K ohm output resistor will yield 1.6 volt, which would be considered a significant signal. Sensitivities better than 10 cells/ml could be achieved by increasing the sample size (from 200 to 500 ml).

Detection thresholds for viables could be similarly estimated.

Rate of growth studies for E. coli and S. marcescens, under the present contract, indicate that a 4 to 5 fold increase in bacterial numbers can be achieved after a 2 hour incubation at 37°C. If the detection threshold is 10 cells/ml (for a 200 ml sample), an unincubated sample containing 2 to 3 cells/ml should, under ideal conditions, be detectable.

While the feasibility of the porphyrin-initiated chemiluminescence technique as a means of sensing low levels of E. coli and S. marcescens has been demonstrated, extension of these studies to cover a wider spectrum of organisms likely to be encountered in spacecraft water systems (e.g., Pseudomonas, Proteus, etc.) is recommended. Since the chemiluminescence response is species dependent, the sensitivities derived for E. coli and S. marcescens may not be representative of the sensitivity achieved for these other organisms.

Some evidence obtained on the present program indicates that the sensitivity for total cells (viable + non-viable) does not change appreciably if the organism is stored at either refrigeration (at ~5°C for 1 week) or ambient (24 hrs) temperature. However, the effect on sensitivity (toward total and viable cells) of exposing these bacteria to the high temperature stress of the water reclamation process should be more thoroughly evaluated and preferably with the chemiluminescence monitor located in close proximity to the water reclamation system.

To achieve maximum sensitivity in detection of bacterial contamination passing through a water regeneration system, the optimum location for the present water monitor would be between the detoxification module (i.e., ion exchange and activated charcoal columns) and the heated storage tanks*. If the latter are maintained at pasteurization temperature, bacteria entering these tanks would be highly stressed, if not completely killed or lysed (the indirect evidence for toxin formation may thus be lost). Growth in the heated storage tanks is unlikely unless thermophiles are present; however, no evidence for the presence of thermophiles was found in the 60-day manned test conducted by McDonnell-Douglas in 1968*.

The recommended manner for using the present system for detection of total and viable organisms is to monitor for total number on an intermittent basis. If a signal above a pre-set threshold is obtained, the run is repeated to see if the baseline signal increases on incubation.

With respect to the minimum sensitivity requirements for an operational water monitor, consideration should also be given to establishing values which are more realistically attuned to the state-of-the-art of current water regeneration systems, particularly with regard to the total (viable + non-viable) cell content. In view of the fact that all eleven of the regenerated water samples received for analysis had total cell contents in excess of 10^3/ml and the fact that potable city tap water also generally run in excess of this number, the requirement for < 10 total cells/ml may be unrealistic.

Although the operational performance of the present instrumentation has been quite reliable, any effort at improvement should be directed toward developing a miniature positive displacement pump for fluid transport which possesses a maintenance-free life in excess of 200 hours, and a filtering system for concentrating the samples that can operate for the same period without requiring filter replacement.

Complete detail and assembly drawings of the recommended version of the water monitor have been issued in a separate miscellaneous report.

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*McDonnell-Douglas Astronautics Co., "60-day Manned Test of a Regenerative Life Support System with Oxygen and Water Recovery", Dec. 1968, NASA CR-98500, DAC 62295.

APPENDIX A

OPERATIONAL SEQUENCE FOR OBTAINING VIABLE AND TOTAL CELL COUNT USING MULTIINCUBATOR APPROACH

(See Figure 1)

1. With reagents in position, turn bench power and air on.

2. Turn on recorder and Premix pump (P 5).

3. Fill lead lines with nutrient and 2M urea by turning on respective pumps (P 2 and P 4). Turn pumps off when each of these fluids have passed through the pumps.

4. Run fresh filtered distilled water (using the sample reservoir inlet and pump P 1) through the system for about 20 min. without the membrane filters (Filter No. 1 and 2) being in position.

5. Place membrane filters in filter holders.

6. Run filtered distilled water through system for about 20 min. reversing the direction of flow (by activating 4-way valves V 1 and V 2) approximately every 5 minutes.

7. Run a 10 ml water blank using the protocol in Table 1, Section 2 (for total-unincubated cycle).

Having established that the system is clean the viable (incubated) cycle is run before the total (unincubated) cycle for establishing sterility of a water sample.

For Incubated Sample

8. Place new incubator tube in position.

9. Run sterile water through incubator (to displace air from incubator) for 10 minutes.

10. Run 10 ml water blank (using protocol shown in Table 1, Section 2) to check cleanliness of incubator.

11. Place bacterial suspension in position and concentrate 100 ml of sample (at 4 ml/min), wash (3 min) and backwash (3 min) with nutrient. This centers the bacterial challenge in the incubator. Close 3-way valves and remove tube for incubation outside the system (37°C - 2 hrs).

While the sample is being incubated, the monitor is used for running a total cell count as described below. Prior to this clear specimen line by repeating step 6.

For Unincubated Sample

12. Repeat steps 8 through 11 except that the tube is not removed for incubation but processed for signal according to the sequence shown in Table 1, Section 2. Replace incubator at end of cycle and replace with incubated sample. Clear specimen line by repeating step 6.

Processing of Incubated Sample

13. Flush by-pass line on incubator tube with 2M urea (Pump No. 4) to displace air. Flow for about 5 min. reversing flow once to vent air to waste.

14. Turn by-pass valves on, incubator tube off, and continue with step 4 (Table 2, Section 2) to the end of the cycle.

In shutting the system down for the day, remove the filter membranes and pass 8M urea through the sample and nutrient lines.

The operational sequences for pumps and valves in the total and viable cycles (Table 1 and 2, Section 2) are illustrated diagrammatically in Figures A-1 and A-2 which follow.

OPERATION

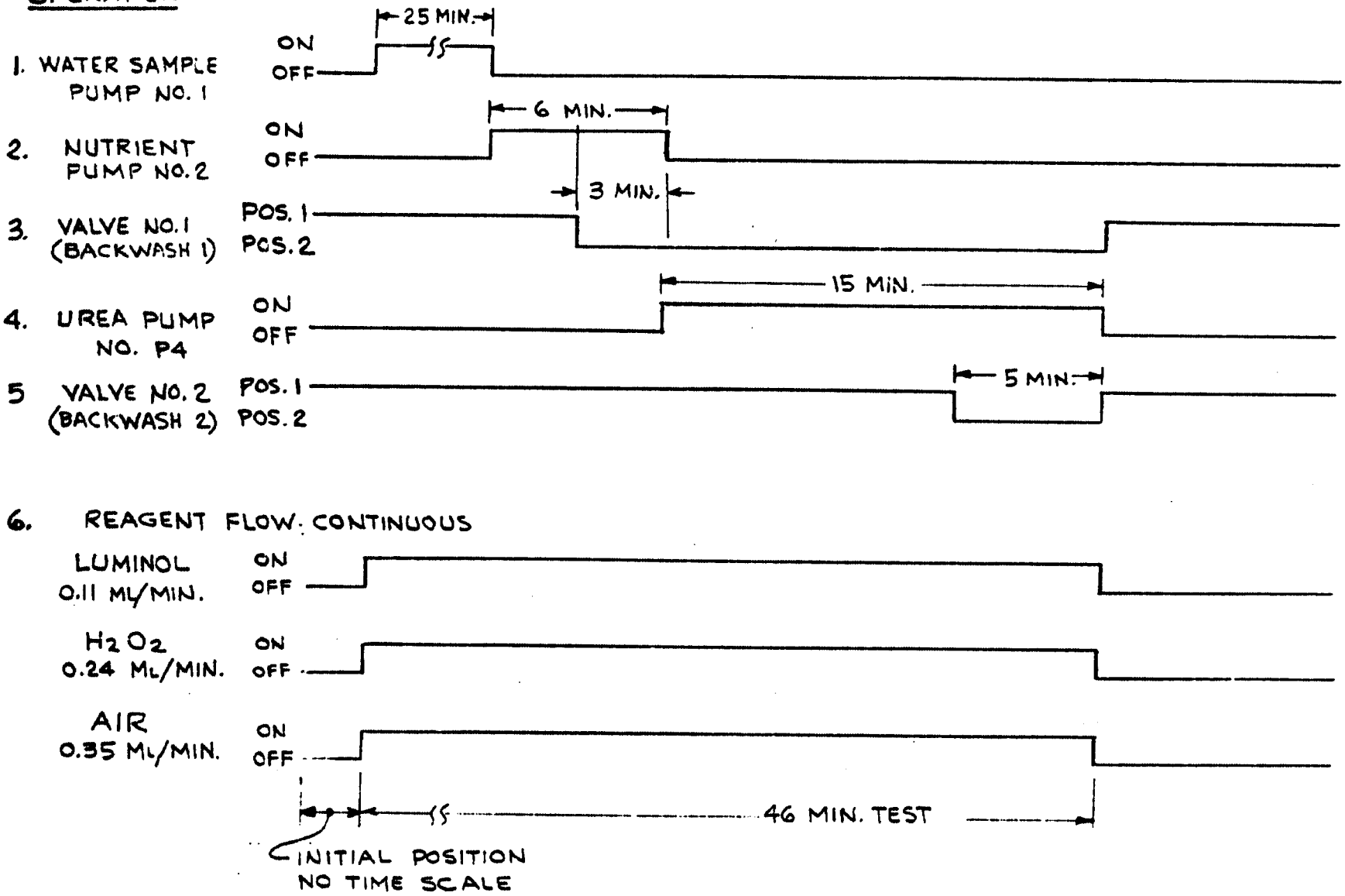

FIG. A1

OPERATIONAL SEQUENCE FOR OBTAINING TOTAL CELL COUNT

(UNINCUBATED SAMPLE, STAGE 3)

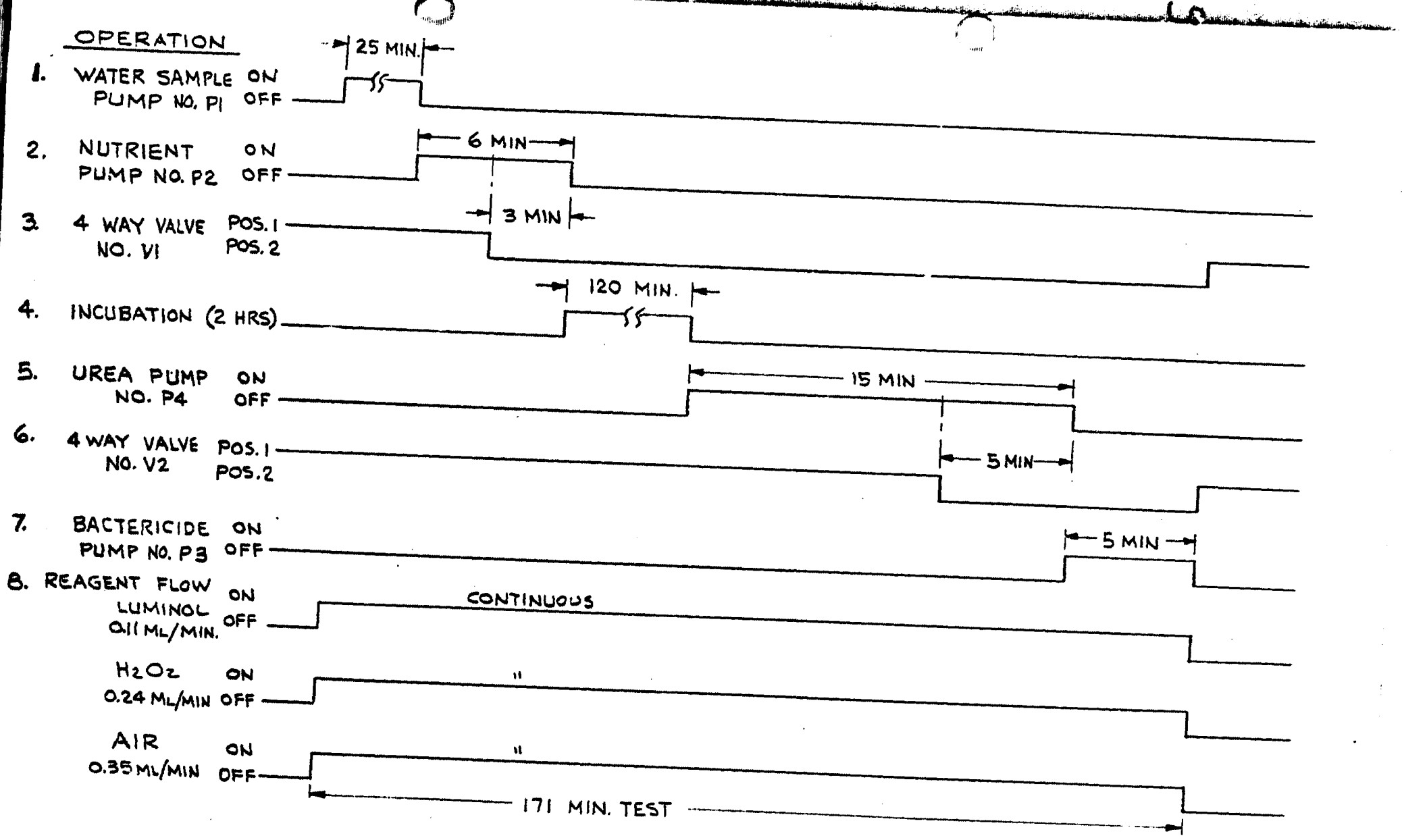

FIG. A2

OPERATIONAL SEQUENCE FOR VIABLE CYCLE
(INCUBATED SAMPLE)

APPENDIX B

REAGENT PREPARATION

A. Dextrose Nutrient

1. Add 23 gm of Dextrose broth to 1000 ml of filtered distilled water.
2. Heat and stir until dissolution is complete.
3. Filter the entire volume successively through
   a. #3 Whatman
   b. #30 Whatman
   c. 0.8 μ Millipore AABP
   d. 0.4 μ Millipore HAWP
   e. 0.22 μ Millipore GSWP
4. Dispense 100 ml of the filtered broth into a particle-free AGI (all glass impinger).
5. Plug the AGI outlets.
6. Autoclave @ 15 lbs for 20 minutes.
7. Use as prescribed.

B. Pour-Plate Media

1. Add 24 gm of tryptone glucose extract agar to 1000 ml of filtered distilled water.
2. Heat and stir almost to boiling until dissolution is complete.
3. Pour into 4 250 ml flasks and stopper with foam plugs.
4. Autoclave @ 15 lbs for 15 minutes.
5. Store @ 55°C until use as prescribed.

C. 8M Urea

Reagent grade (Baker analyzed) urea is dissolved in glass-distilled water to a final concentration of 8 molar. The solution is filtered through

a 0.1 u Ultripor filter and then passed through a mixed bed ion exchange column (equal quantities of Dowex AG 50W-X8 and Dowex AGI-X8, 200-400 mesh, 200 ml bed volume). The pH of the effluent from the column is adjusted to pH 7.0 to 7.5 with hydrochloric acid. Filter through a 0.2 μ (prewashed*) Millipore prior to use.

D. 2M Urea

An appropriate dilution of the 8M urea is made with glass distilled water and the solution filtered through a 0.2 μ pore sized filter before use.

* Pass 500 ml of distilled water through Millipore prior to use to remove finishing agent from filter.